REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
02-19-2008	Final	May 2006 to May 2007

4. TITLE AND SUBTITLE

THE KEY INVOLVEMENT OF POLY(ADP-RIBOSYL)ATION IN DEFENSE AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES

5a. CONTRACT NUMBER

5b. GRANT NUMBER
FA9550-06-1-0412

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Anton Vila-Sanjurjo & Carlos Bustamante

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

MCB Department
University of Califonia Berkeley
717 Potter st, room 177
Berkeley, Ca 94720

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR/NL
875 N RANDOLPH ST, STE 325
ARLINGTON, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution A: Approved for Public Release

AFRL-SR-AR-TR-08-0152

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our study during the period 2006-2007 focused in the creation of a minimal synthetic organism by reversing the endosymbiosis of mitochondria.

20080331090

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Anton Vila-
X				39	19b. TELEPHONE NUMBER *(include area code)* 510-486-6839/510-643-9706

Reversing the Endosymbiosis of Mitochondria: Development of a Transcriptionally independent Mitochondria.

Summary of Progress Report

During the first year of DARPA funding we have accomplished major progress towards achieving the four goals described in our original proposal. In particular, we have demonstrated that it is possible to deliver DNA to mammalian mitochondria within living cells, this being the major obstacle towards the transformation of these organelles. As shown below, we are also well in our way to achieve all other three goals. We are particularly excited about the progress towards accomplishing our most challenging goal, i.e. building a transcriptionally independent mitochondria. Following is a summary of all these achievements that serves as an introduction to our progress report.

First Goal: Delivery of Exogenous DNA to the Mitochondrion

We show below that we can target DNA oligonucleotides to mitochondria of mouse cells by means of two methods, namely protofection (Khan, and Bennett, 2004) and DQAsome-mediated delivery of DNA (Weissig, *et al*, 2006) (see **Figures 9A** and **9B**). In order to do this we had to first design and synthesize protofection protein vehicles (see section entitled "PROTOFECTION" below) as well as familiarize ourselves with DQAsome vesicles by visiting the Weissig lab at Northeastern University, Boston, MA. (see section entitled "DELIVERY OF DNA TO MITOCHODRIA BY TRANSFECTION REAGENTS AND VESICLES" below). We are currently attempting to understand whether the DNA oligonucleotides actually enter the mitochondrial matrix.

Clearly, the next step is to be able to deliver and express a bigger piece of DNA. In preparation for this, we have synthesized a number of mitochondrially encoded reporter genes (see section entitled "MT-REPORTERS"). We are in the process of targeting these constructs to the mitochondria of mouse cells.

Although we did not initially proposed yeast as one of our model organisms for mitochondrial research, we have decided to pursue research with this organism for the following reasons:

-DNA can be delivered to yeast mitochondria by biolistic transformation.
-Homologous recombination is active in yeast mitochondria.
-Numerous mitochondrial mutants exist hat can be used in further manipulations.
-Large number of genetic and molecular tools are available to this system.
-Easy generation of nuclear knockouts.

Although initially our lab was not familiarized with the manipulation of yeast, yeast mitochondrial genetics, and biolistic transformation of yeast mitochondria, we have spent part of the year learning all these techniques (see section entitled "MT-TRANSFORMATION: FAMILIARIZATION AND OPTIMIZATION" below). This experience has greatly expandedthe scope of the lab in mitochondrial research.

Second and Third Goals: "Testing the Plasticity of the Mitochondrial Genome" and "Testing the Transcriptional Activity of the New Promoters".

The task of inserting exogenous genes in the highly compact, circular mammalian mitochondrial genome, requires finding appropriate spots where gene insertion does not interfere with normal mitochondrial function (see section entitled "**TESTING THE PLASTICITY OF THE MAMMALIAN MT-GENOME**" below). We have built a number of constructs in which we have inserted the elements necessary for the expression of reporter genes in the mitochondrial matrix "landing pad" (see **Figure 5** below). The performance of these constructs in the mitochondrial matrix and the expression of the reporter genes will be tested once a method of mitochondrial transformation is reproducibly established.

Fourth Goal: Making a Transcriptionally Independent Mitochondrion

Mammalian front: Once we have verified that synthetic mitochondrial genomes carrying a "landing pad" and that exogenous genes can be expressed from this region, we will proceed to insert a synthetic gene encoding the mitochondrial RNA polymerase, as described in "TESTING THE PLASTICITY OF THE MAMMALIAN MT-GENOME" below.

Yeast Front: Together with GenScript Inc., we have designed and built constructs carrying the gene for the yeast mitochondrial RNA polymerase, RPO41, that is compliant with the mitochondrial genetic code (see section entitled "Design and construction of a transcriptionally independent yeast mitochondrion" below). Given the high bias towards AT rich sequences in the yeast mitochondrial genome, it took more than 4 months for the synthetic gene to be made and delivered to us. Since then we have cloned the gene in the appropriate vectors and started the biolistic transformation of a yeast RPO41 knockout strain that we have also made. We feel that at this point we are very close to achieve the first step towards the goal of making a transcriptionally independent mitochondrion. Once the insertion of RPO41 in the mitochondrial genome is confirmed and the activity of its encoded protein demonstrated, we will proceed to complete this goal by inserting the unique transcription factor in yeast mitochondria, MTF1.

We are extremely excited about the progress we have made and also about the new avenues of investigation that are opening before our eyes. However, we are also aware of our limitations, mainly regarding the insufficient number of researchers we currently have. All the research in this report has been performed by one postdoc, three grad students (two of them present only for part of the current year), one technician (present only for a few months), and several undergrad students. While we believe that the progress we have made is spectacular, specially if one considers the difficulty and the novelty of this research, we also feel that we need to substantially increase the number of researchers involved in this project. Our goal is to hire at least one permanent technician, two more postdocs, and two more grad students. For this reason we are requesting the funds to maintain the current lab activity and support for the proposed increase in our research capacity.

What follows is a detailed description of the purpose of this project and the progress obtained within the first year of DARPA funding.

Reference List

Khan, S.M., and Bennett, J.P.,Jr. (2004). Development of mitochondrial gene replacement therapy. J. Bioenerg. Biomembr. *36,* 387-393.

Weissig, V., Boddapati, S.V., Cheng, S.M., and D'Souza, G.G. (2006). Liposomes and liposome-like vesicles for drug and DNA delivery to mitochondria. J. Liposome Res. *16,* 249-264.

Creating a minimal organism by Reversing Mitochondrial Endosymbiosis

Progress report August 2007

Carlos Bustamante & Anton Vila-Sanjurjo
MCB Department, University of California, Berkeley
Berkeley West Biocenter
Lawrence Berkeley National Laboratory
Berkeley, CA 94720

SPECIFIC AIMS

The final goal of this project is to build a minimal, autonomous cell based on a mitochondrion. The immediate goal is to create a transcriptionally independent mitochondrion. Two parallel lines of investigation, involving mammalian and yeast mitochondria, are currently in progress in the lab. The main reason behind this parallel experimental approach is that while both organisms have specific advantages relevant to the work proposed here, neither one emerges as a superior model system at this stage of the research. Moreover, learning to manipulate the mitochondrial (mt) genome in either system is highly desirable, as *S. cerevisiae* is the fundamental eukaryotic "chassis" considered in the emerging discipline of Synthetic Biology and mouse is a model organism for human disease. The two specific aims proposed here are:

1) Creation of a transcriptionally independent mouse mitochondrion.
2) Creation of a transcriptionally independent yeast mitochondrion.

INTRODUCTION

MINIMAL ORGANISM

What is the minimum complexity required to sustain life? Answering this question ultimately will lead to the discovery of the laws that govern the living state. A living cell is a complex system that is capable of performing all the central process unique to life: self assembly, energy generation, self replication, division, and most importantly, the coordination of all these functions into a stable entity. *Mycoplasma genitalium* is the free-living cell with the smallest genome known to date (only 525 genes) (Glass, *et al*, 2006). However, it has been estimated that the minimum gene set required to assemble a stable living cell may contain as few as 206 genes (Gil, *et al*, 2004). Other estimates, however, are more conservative, with a number of essential genes ranging from 250 to 430 genes (Hutchison, *et al*, 1999; Kobayashi, *et al*, 2003; Mushegian, and Koonin, 1996). Recently, *Carsonella ruddii*, a γ-proteobacterial symbiont that appears to be present in all species of phloem sap-feeding insects, was reported to contain only

182 open reading frames (Nakabachi, *et al*, 2006). Even though this gene set constitutes the smallest genome known for an organism, it seems insufficient for supporting autonomous bacterial life. Therefore, the genome of *C. ruddii* cannot be considered a minimal genome.

CONSTRUCTION OF A MINIMAL ORGANISM BY REVERSING MITOCHONDRIAL ENDOSYMBIOSIS

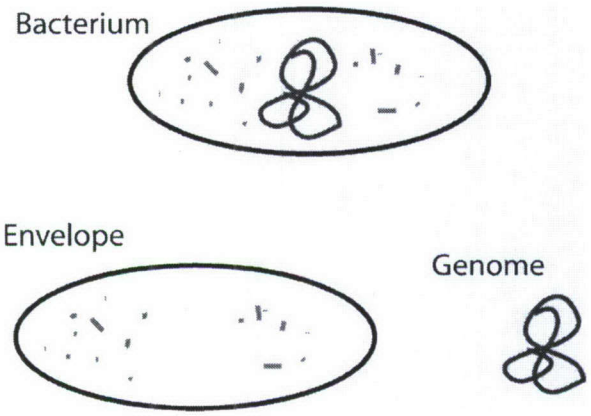


Figure 1

For the purpose of this analysis, we can think of a cell as a system consisting of an envelope and a genome. The envelope is the scaffold that contains and includes all the cellular functions with the exception of the genome itself, which consists of the gene set that encodes all the components of the envelope (**Figure 1**). How does one design and build a minimal cell in the laboratory? The simplest approach would be to follow a top-down strategy where an already simple system, such as *Mycoplasma*, is simplified to its minimum complexity. In this approach, the complexity of a naturally occurring genome/envelope combination is reduced by systematic deletion of unnecessary genes. This approach has the advantage that it requires minimal synthesis and design. However, these are also its main limitations. First, while the deletion of certain genes may not be lethal at the individual level, multiple deletions could have unexpected effects. Second, different organisms may have evolved alternative solutions to solve a particular biological problem. Thus, it is possible that metabolic pathways simpler than the ones conserved in modern *Mycoplasma* exist in other organisms. In agreement with this, the number of essential genes estimated from transposon mutagenesis studies performed in *M. genitalium* is 430 genes (Glass, *et al*, 2006). This number corresponds to 82% of the *M. genitalium* genome and it is far from the theoretical estimates of 206 to 250 genes (Gil, *et al*, 2004; Mushegian, and Koonin, 1996) or from the core of 279 essential genes identified by mutagenesis of *Bacillus subtilis* (Kobayashi, *et al*, 2003), indicating that this bacterium could be a better start point than *M. genitalium* for a reduction in genomic complexity. Finally, while an enormous amount of knowledge can be obtained from this top-down approach, it will not represent a milestone towards the goal of designing wholly synthetic organisms in the laboratory.

Alternatively, one could think of a bottom-up approach in which the minimal organism is built starting from scratch, e.g. artificial cells composed of a minimal genome and a minimal envelope made from vesicles containing precisely defined constituents. While the construction of simple artificial envelope/genome systems has been attempted (Luisi, *et al*, 2006; Noireaux, *et al*, 2005), building an artificial minimal envelope, that allows all essential cellular functions to proceed in a manner that supports the growth of a synthetic organism, may prove extremely difficult.

We propose a variation of the bottom-up approach by using a naturally occurring

envelope within which we will build a minimal genome, so that the combination of the two behaves as a self-replicating minimal bacterium. This approach will allow us to arrive at a rationally designed as opposed to evolutionarily derived minimal cell. To this end, we have chosen a mitochondrial envelope as the house for our synthetic, minimal genome. Mitochondria are the descendants of an alpha proto-bacterial ancestor that was presumably engulfed by either an archaeal or a primitive eukaryotic host as part of an endosymbiotic event that occurred about 2 billion years ago (Gabaldon, and Huynen, 2004; Osteryoung, and Nunnari, 2003). During evolution, a large fraction of the mt-genome was transferred to the host nucleus. Modern mitochondria have their own discrete, quasi-autonomous genome and their own protein synthesizing machinery. The modern mt-genome carries a limited number of genes that always includes two rRNA genes and, in most cases, 20 or more tRNAs (Scheffler, 1999). In addition to these genes, mammalian mitochondrial DNA (mtDNA) contains 13 genes for proteins of the oxidative phosphorylation complex, while yeast carries 9 such genes plus a single gene encoding a mitochondrial protein (Scheffler, 1999). Given that the number of human mitochondrial proteome is estimated in more than 1,000 proteins and that of yeast in about 500 (Andreoli, *et al*, 2004), these mitochondria are examples of envelopes almost completely devoid of their genomes.

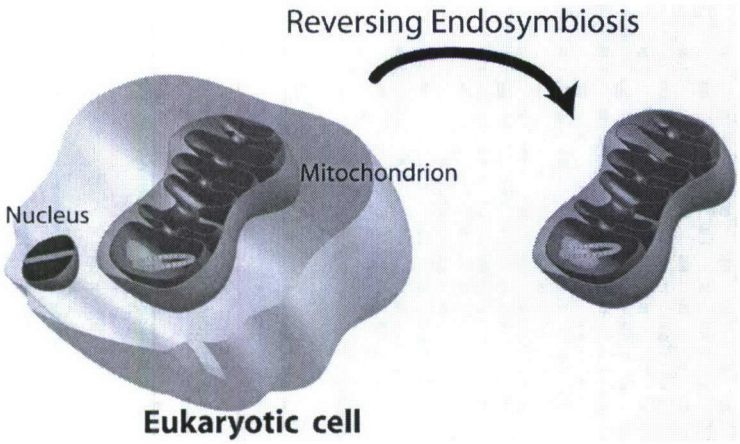


Figure 2

Specifically, we propose to use mitochondria as a platform to construct a minimal, viable set of genes that will eventually result in an envelope-genome combination that is no longer dependent on the cell nucleus (**Figure 2**). This goal will be achieved through a stepwise transfer of the genes needed to confer autonomy to the new cell. The chosen set of genes will be modified to comply with the mitochondrial genetic code prior to transfer. Following transfer, the nuclear copies of these genes will be deleted from the genome. At every step, nature will dictate the viability of the new envelope-genome combination by the ability of the modified organelle to survive within its host. **The final goal of this endeavor is to build a cell whose envelope is based on a mitochondrion and whose genome is synthetically designed, so that the genome-envelope combination results in a minimal cell capable of surviving and dividing outside of its original host.**

TRANSCRIPTIONALLY INDEPENDENT MITOCHONDRIA

The initial goal of the project is to expand the mt-genome with the genes necessary to construct a transcriptionally independent mitochondrion. Defining the transcriptional machinery that will be responsible for the transcription of the new genome is a critical step. Since transcription, replication, and maintenance of the mt-genome are closely linked processes (Alam, *et al*, 2003; Kanki, *et al*, 2004; Lecrenier, and Foury, 2000; Xu, and Clayton, 1995), it

appears reasonable to use the existing mt-transcriptional machinery while constructing the new cell. This approach will avoid the complete redesigning of the existing mt-genome.

The mt-genomes of yeast and mammalian cells are transcribed by nuclear-encoded RNA polymerases (POLRMTs) resembling those of bacteriophages T7, T3, and SP6 (Tiranti, *et al*, 1997). In addition to the polymerase, transcription in yeast requires a single transcription factor mtTFB (Tracy, and Stern, 1995), whereas this process in mammals requires two similar factors TFB1M and TFB2M along with the structurally unrelated TFAM protein (Falkenberg, *et al*, 2002).

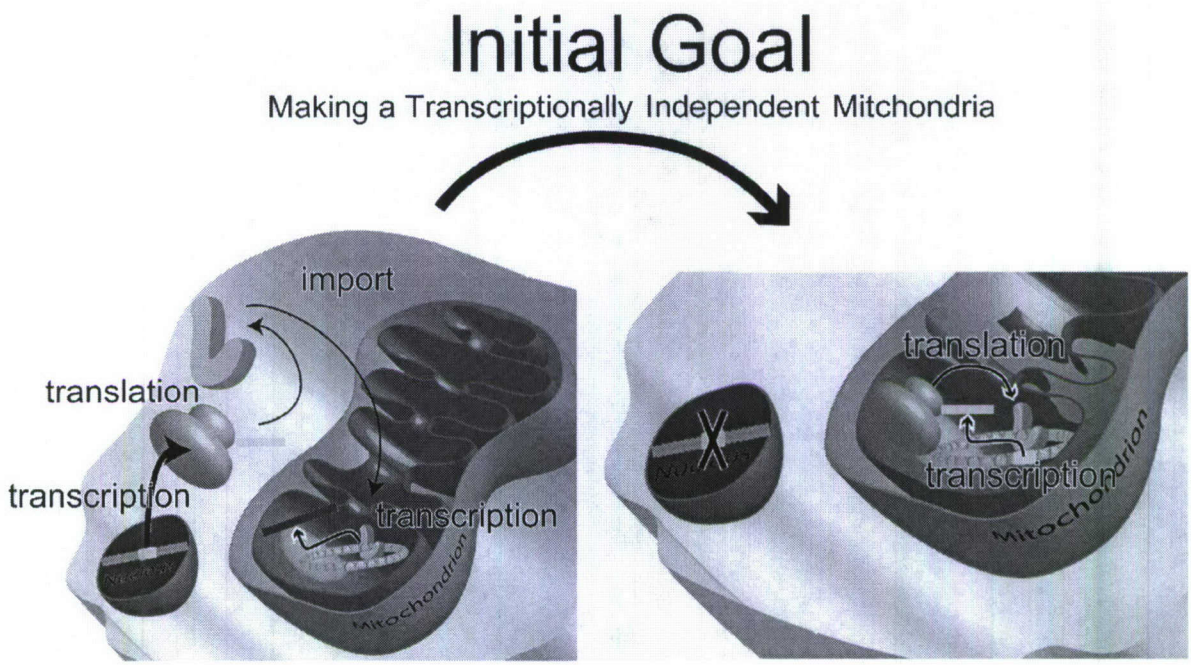


Figure 3

Our strategy is schematically summarized in **Figure 3**. The vast majority of mitochondrial proteins are encoded by genes contained in the nucleus (red half sphere). These include the proteins composing the mt-transcriptional machinery, i.e. the POLRMT and the mitochondrial translation factors. The gene encoding POLRMT is shown in yellow in **Figure 3** (left panel). In a normal eukaryotic cell, the POLRMT mRNA (yellow, thick line) is translated by cytoplasmic ribosomes (cyan double oval), resulting in the synthesis of POLRMT (L-shaped, cyan object). POLRMT is then imported into mitochondria where it transcribes the mt-genome (helical ring). In the right panel of **Figure 3**, a version of the POLRMT gene, recoded to comply with the mt-genetic code, has been introduced into the mt-genome (yellow square). At the same time, expression from the nuclear gene has been abolished (cross sign). In this modified cell, the mt-encoded POLRMT drives the expression of the mt-genes.

PRELIMINARY WORK AND FUTURE DIRECTIONS

Any attempt to expand the coding capacity of the mt-genome requires the ability to deliver exogenous genes to the mt-matrix and to stably place these genes in the mt-genome. For this reason, we have spent most of the period of DARPA funding developing techniques to achieve these goals. Two model organisms have been chosen based on the considerations shown in the following table (listed in red are some obvious disadvantages of either model system):

Yeast	Mouse
The model organism for mitochondrial research.	Model organism for the study of mammalian mitochondrial physiology and mitochondrial diseases.
DNA can be delivered to yeast mitochondria by biolistic transformation.	It is possible to clone the entire mouse mt-genome (17kb) in E. coli.
Homologous recombination in mitochondria.	Techniques for creating stable knockouts of the two nuclear alleles of the mitochondrially-targeted gene exist (Chuang, *et al*, 1999).
Numerous mitochondrial mutants.	No "reasonably" proven methods to deliver DNA to mitochondria.
Large number of genetic and molecular tools.	The existence of mt-homologous recombination is a matter of debate.
Easy generation of nuclear knockouts.	
Large mt-genome, 85Kb.	

Based on our choice of model organisms, we have laid out the following two specific aims. These are:

1) Creation of a transcriptionally independent mouse mitochondrion.
2) Creation of a transcriptionally independent yeast mitochondrion.

What follows is a description of the progress obtained by the lab on these two specific aims during the period of DARPA funding.

Specific Aim 1: Creation of a transcriptionally independent mouse mitochondria.

Two different lines of work are described in this section. The goal of the first one, entitled "Testing the Plasticity of the Mammalian mt-genome", is to redesign part of the mouse mt-genome so that its coding capacity can be expanded. The second line of work, entitled "Delivery of DNA to the Mouse Mitochondria", is aimed at testing and developing methods for the transformation of mitochondria in mammalian cells.

TESTING THE PLASTICITY OF THE MAMMALIAN MT-GENOME

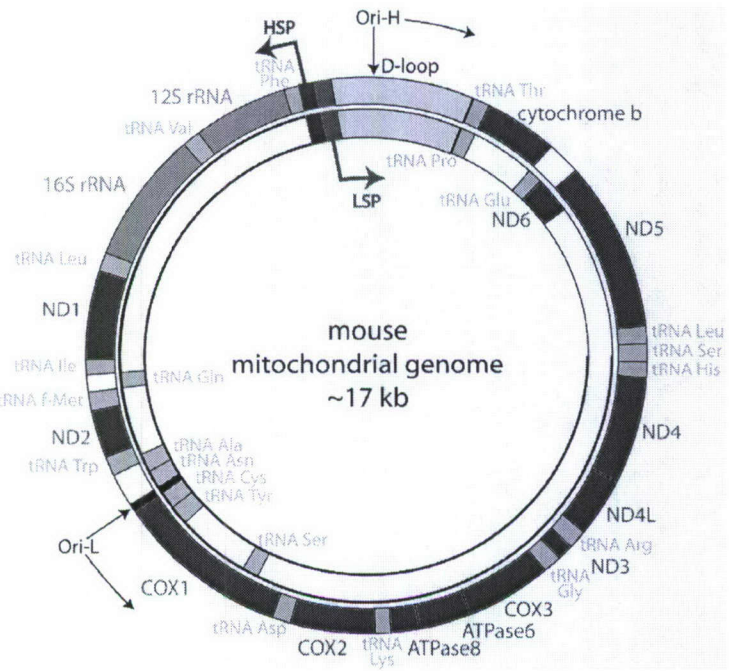


Figure 4

Because the two strands of the mt-genome differ in their GT content and can be separated by centrifugation in denaturing gradients, they have been named heavy (outer circle in **Figure 4**) and light strands (inner circle in **Figure 4**). Both strands serve as templates for polycystronic transcripts almost as long as the genome itself. In order to use this genome to build our synthetic cell, one has to define where new genes will be inserted. While the insertion of a few genes within the two polycistronic units could be attempted, the use of this approach for the construction of a whole new genome with few hundred genes does not seem a viable solution. Moreover, we foresee the use of promoter strength as a means to regulate the relative expression of new genes. Therefore, the use of additional promoter units for the expression of the new genes is probably the most rational choice. However, finding a region where the insertion of these transcription units does not result in the disruption of normal genomic function is a challenging task. This is due to the small size of the mouse mt-genome (16.6 kb), which results in a highly compact arrangement of its 37 genes (for current reviews on the organization, replication and transcription of the mammalian mt-genome see Bonawitz, *et al*, 2006; Fernandez-Silva, *et al*, 2003). Only two non-coding regions exist in mammalian mtDNA (**Figure 4**). One such region is the so called "D-loop" (yellow in **Figure 4**), located between the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. This region contains the origin of replication for the heavy strand (Ori-H in **Figure 4**) and the two transcriptional promoters HSP and LSP (for Heavy and Light Strand Promoter, respectively). The other region is a ~30 nucleotide segment that contains the origin of replication for the light strand (Ori-L in

Figure 4). Because these two regions contain regulatory elements for both transcription and replication, their disruption by the insertion of exogenous DNA could seriously compromise the integrity of the genome.

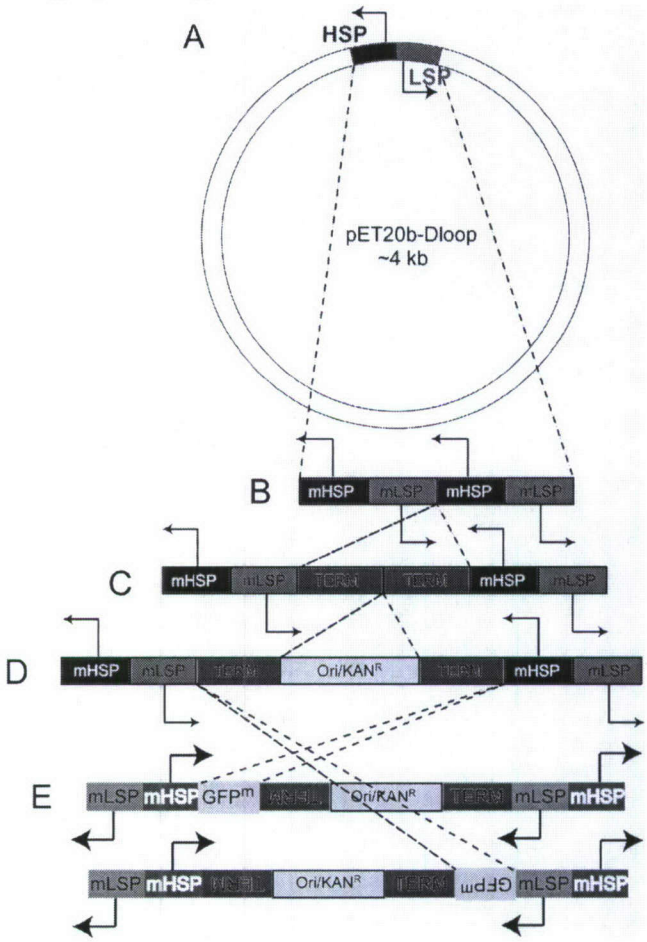


Figure 5

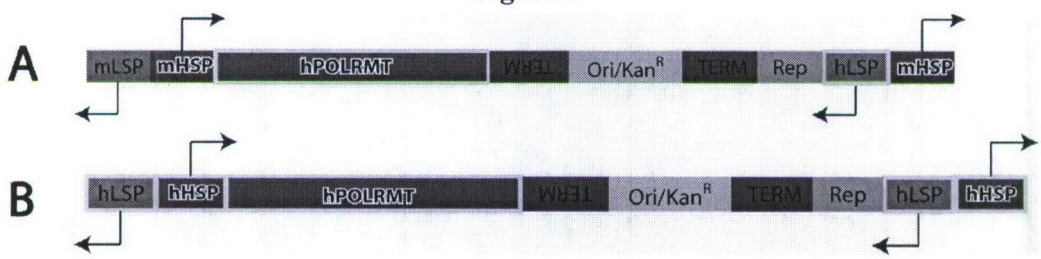
In order to expand the coding capability of the mammalian mt-genome without interfering with the existing transcriptional and replicational arrangement, we decided to use the region between HSP and LSP to insert exogenous genes. The two promoter regions are physically adjacent to each other (**Figure 4**) and their sequence requirements have been thoroughly studied *in vitro* (Chang, and Clayton, 1984; Chang, and Clayton, 1986a; Chang, and Clayton, 1986b; Chang, and Clayton, 1986c; Fisher, *et al*, 1987; Gaspari, *et al*, 2004). Both promoters consist of a consensus sequence motif of 15 bp surrounding the initiation points and an enhancer element located at a precise distance upstream. The upstream element contains the binding site for the transcription factor TFAM. While HSP and LSP are functionally independent *in vitro* (reviewed in Fernandez-Silva, *et al*, 2003), it is possible that the spacing of the two promoter units relative to one another might have an unknown role *in vivo*. For this reason, we have designed a series of genomic constructs that include additional LSP and HSP promoters where the spacing and primary sequence between each LSP and its adjacent HSP is left intact. We have taken advantage of the fact that the mouse mt-genome has been cloned as a plasmid in *E. coli* (Yoon, and Koob, 2003). The modified genomic constructs are shown in **Figure 5**. Initially, the region containing the D-loop was cloned into vector pET20b (LSP and LSP-like promoters in green, HSP and HSP-like promoters in blue) (**Figure 5A**). To this construct, we added one extra copy of the HSP/LSP promoter unit (**Figure 5B**). Note that the spacing between HSP and LSP in both promoter units is identical to that found in wild type mouse mt-genome. A restriction site at the junction between the original and the new promoter units can be used to open this region for the insertion of new DNA, e.g inverted mt-transcriptional termination units (red rectangles in **Figure 5C**) (Asin-Cayuela, *et al*, 2005; Fernandez-Silva, *et al*, 1997; Martin, *et al*, 2005). The promoter units firing inward will be used to direct the transcription of our genes of interest, while those firing outward will control the normal transcription of the mouse mt-genome. The "inward/outward" promoter nomenclature will be used hereon to distinguish the two sets of promoters present in the modified mt-genome. In addition to the mt-sequences, an *E. coli* origin

of replication and a kanamycin resistance gene were inserted between the two inverted terminators (yellow rectangle in **Figure 5D**) to allow propagation of the final constructs in *E. coli.* At this point we considered that the construction of the "landing pad" for the insertion of exogenous genes was finished. Additional cloning steps resulted in the addition of GFP reporter genes that comply with the mouse mt-genetic code (light green rectangle in **Figure 5E**). Once these constructs are inserted into an otherwise intact mt-genome and introduced in the mt-matrix, they will allow us to monitor the activity of the inward promoters *in vivo.* This will require the use of one of the methods for mt-transformation described below.

Success at this stage will determine whether we will proceed to the next step, that is, the addition of the genes that will endow mouse mitochondria with transcriptional independence. Given that, to the extent possible, we will be using POLRMT for the expression and regulation of the genes in the new mt-genome, we are planning on performing extensive functional and structural characterization of this enzyme and its associated factors, both *in vivo* and *in vitro,* to complement the knowledge available in the literature. One major goal is to understand how to use promoter strength to regulate gene expression. We will test promoter strength in vivo by mutagenizing the sequence of the inward HSP and LSP promoters of **Figure 5E** followed by measurement of their activity as a function of reporter output. In addition, as part of a collaboration with the laboratory of Lourdes Dominguez-Gerpe at the University of Santiago de Compostela in Spain, we are attempting to isolate recombinant forms of the POLRMT and its associated transcription factors, to pursue their biochemical and structural characterization (see "Protofection" section below).

The initial goal of the project is to build a transcriptionally independent mitochondrion, which will require the insertion of the genes for the transcriptional machinery in the "landing pad" of our synthetic mt-genome. A scheme of the proposed constructs is shown in **Figure 6**. We will take advantage of the strong similarity between the mouse and human mt-genomes (Chang, and Clayton, 1986a; Tracy, and Stern, 1995) and the fact that the transcriptional machinery of

Figure 6



either species is unable to initiate transcription when confronted with promoters from the other species (Chang, and Clayton, 1986a; Chang, and Clayton, 1986b; Gaspari, *et al*, 2004). Interestingly, the determinant of species specificity appears to be the POLRMT itself, meaning that a human POLRMT (h-POLRMT) should be able to work on human promoters in the presence of mouse transcription factors (Gaspari, *et al*, 2004). **Figure 6A**, shows one of the mt-genomic constructs we intend to build. In this construct a mitochondrially recoded h-POLRMT gene (purple) is placed under an inward-firing mouse promoter and a reporter gene is placed

under a human promoter (yellow-framed, inward h-LSP in this example). Expression of the reporter gene in such construct will be a measure of the activity of h-POLRMT. Once the mitochondria are primed with h-POLRMT, all inward and outward mouse promoters will be replaced with their human counterparts **Figure 6B**, thus resulting in a transcriptionally independent mitochondrion. By proceeding this way, we would avoid the creation of the nuclear gene knockouts during these initial steps.

DELIVERY OF DNA TO THE MOUSE MITOCHONDRIA

The success of this project is dependent on our ability to deliver DNA to the mitochondrion and to integrate this DNA into the mt-genome. Inserting DNA into mitochondria in-vivo requires transfer of DNA across three double membranes: the plasma membrane and the outer and inner mt-membranes. At present, no reliable methods for the delivery of DNA to mammalian mitochondria exist, despite the enormous interest in developing such methods for their use in mt-gene therapy (Khan, *et al*, 2007). For this reason, we have focused most of our efforts in developing the technology to achieve this goal. Isolated reports of successful mt-transformation do exist but in no case have the reported results been reproduced. We have used these reports as starting points in our search for a reliable method of mt-transformation. What follows is a description of some of the strategies currently under exploration in the lab.

MT-REPORTERS: To unambiguously establish that a piece of exogenous DNA localizes to the mt-matrix, it is crucial that its expression in this cellular compartment can be monitored. To specifically test for mt-expression of foreign genes we engineered a series of mt-reporters that carry mitochondrially recoded versions of GFP or red fluorescent protein (DsRed) under the control of the mouse mt-promoters. The GFP or DsRed coding region (orange box) in the reporter constructs is preceded by either the mouse HSP (**Figure 7A**) or LSP (**Figure 7B**) plus varying lengths of the regions surrounding the promoters (black line) to ensure that we include all the genomic sequences necessary for proper transcription, mRNA processing, and translation. Also shown, are the primers used for PCR amplification of the different genomic fragments preceding the reporter gene (black arrows in **Figures 7A** and **7B**). The genomic part of the constructs and the primers are aligned to an organizational scheme of the mouse genome for clarity.

All mt-constructs contain a point mutation in the reporter gene that, because of codon usage differences between mitochondrial and nuclear codes, will only give rise to full length proteins when expressed in the mt-matrix. This was done by mutating a unique tryptophan codon UGG to its mitochondrial equivalent UGA, which is a termination codon for cytoplasmic ribosomes (**Figure 7C**). As a result, these constructs should produce non-fluorescent, truncated forms of GFP and RFP when citoplasmically expressed (**Figure 7C**). An example of this is shown in **Figure 7D**. Mouse EPH4 cells (Xu, *et al*, 2007) with DsRed-labeled mitochondria (red in panels 2 and 4, see "Uptake of Isolated Mitochondria" section below for a cell line description), were transiently transfected with GFPmito6NM, which carries a non-mutated version of one of the mt-constructs of **Figure 7B** (lipofectamine 2000 from Invitrogen was used as a transfection reagent). The cells show varying degrees GFP cytoplasmic fluorescence, panels **3** and **4**. Also shown are Hoechst-labeled nuclei in panel **1** (blue), an overlay of all three colors in panel **4**, and a bright field image in panel **5** (Magnification 60X). The generalized pattern of

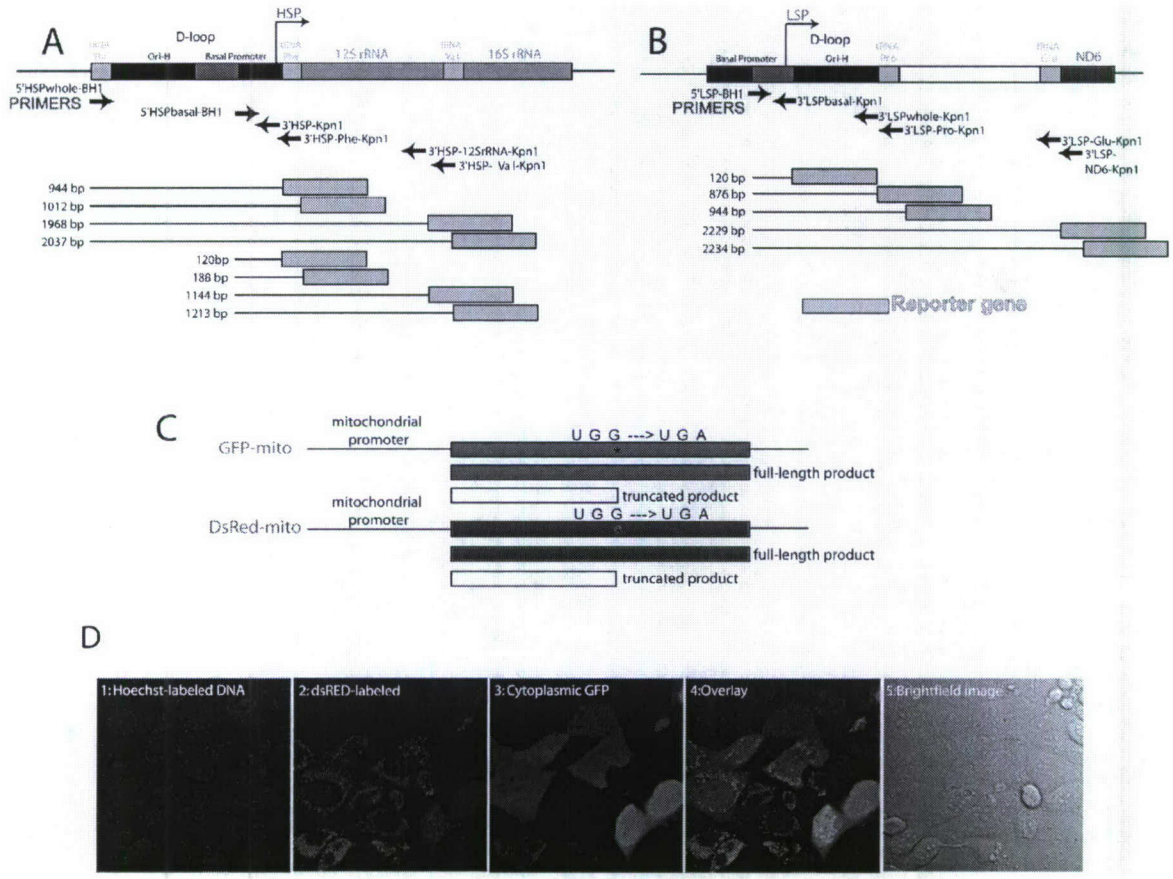


Figure 7

fluorescence observed with GFPmito6NM has not been observed with its equivalent construct GFPmito6, carrying the mitochondrially recoded version of GFP. This result indicates that the mutation indeed results in a non-fluorescent version of the protein when expressed in the cytoplasm. These constructs will be used to test all the methods of mt-transformation described below.

PROTOFECTION: Khan & Bennett (Khan, and Bennett, 2004) have reported that delivery of DNA to the mitochondria of mammalian cells can be achieved by a new method dubbed protofection. The method relies on a previous observation that the Protein Transduction Domain (PTD) of the TAT protein can direct the localization of GFP fusion proteins to the mt-matrix, as long as they carry a mt-localization signal (MLS) in addition to TAT (their construct will be hereon named TAT-MLS-GFP) (Del Gaizo, and Payne, 2003). PTDs are small peptides that have the ability to cross lipid bilayers in a receptor-independent manner. When such peptides are covalently attached to "cargo" proteins, they can deliver these proteins through lipid membranes. The best characterized PTD is the TAT domain of the HIV-TAT protein (Dietz, and Bahr, 2004). MLSs are N-terminal peptides that target proteins to mitochondria. These proteins are delivered through the mt-import pathway consisting of two mt-translocases, the TOM complex in the outer membrane and the TIM23 complex in the inner membrane (reviewed in Neupert, and Herrmann, 2007). Once in the matrix, some MLSs are cleaved by endogenous

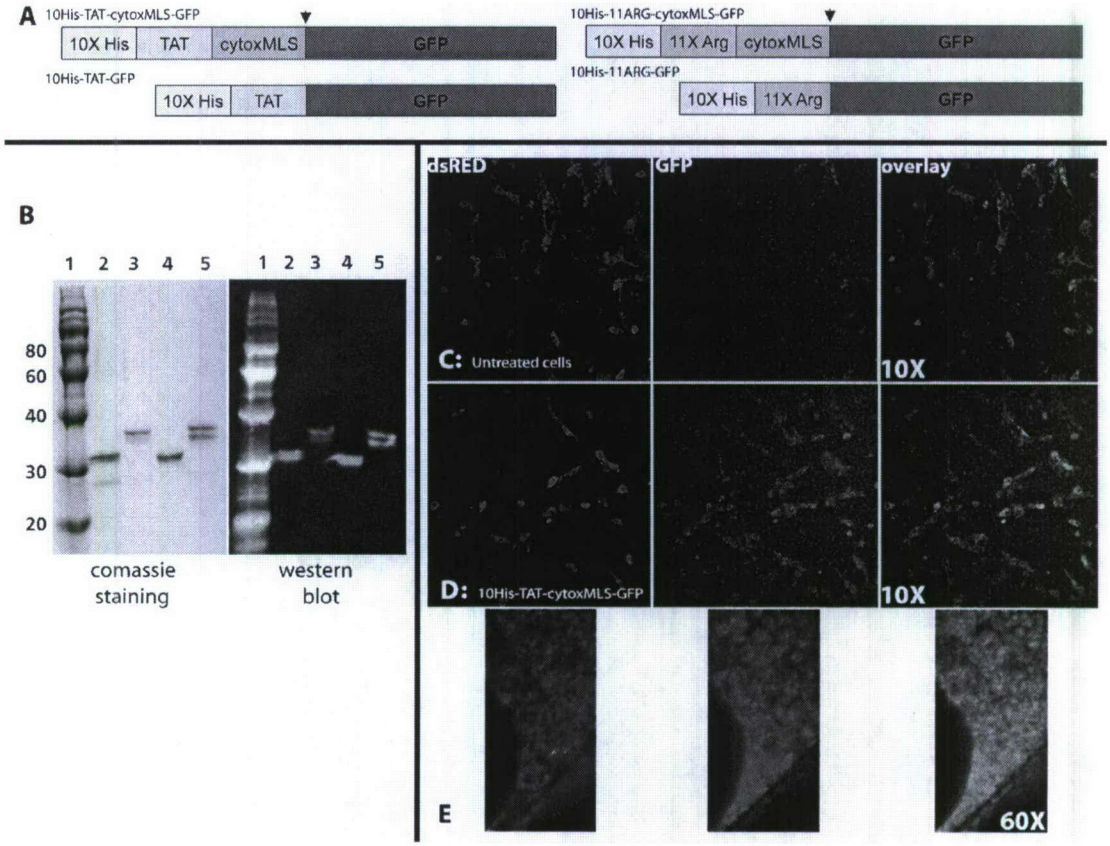


Figure 8

proteases. According to Del Gaizo & Payne (Del Gaizo, and Payne, 2003), both TAT-GFP and TAT-MLS-GFP are able to reach the mt-matrix but only the GFP domain of TAT-MLS-GFP accumulates there after being cleaved downstream of the MLS sequence.

Given the enormous potential importance the protofection method for our project, we have decided to thoroughly test the performance of TAT-MLS fusion constructs in our lab. For this reason, we attempted to re-create the results of Del Gaizo & Payne (Del Gaizo, and Payne, 2003). Since their published TAT-MLS-GFP construct carrying the MLS of the mt-Malate DeHydrogenase (mMDH) (Del Gaizo, and Payne, 2003) has been difficult to purify in our hands, we have made a similar construct by fusing GFP to the MLS of the subunit VIII of the human cytochrome C oxidase (cytox) and to TAT (**Figure 8A**). Similarly to mMDH, CytoxMLS is known to efficiently direct GFP and DsRed to mitochondria *in vivo* (see "Uptake of Isolated Mitochondria" section below) (Rizzuto, *et al*, 1995) and to be cleaved in the mt-matrix (black arrow in **Figure 8A**) (Rizzuto, *et al*, 1989). The construct, named 10His-TAT-cytoxMLS-GFP also carries a run of 10 histidines (10His tag) to allow easy purification by affinity chromatography. In addition to 10His-TAT-cytoxMLS-GFP, we have made all the other GFP constructs shown in **Figure 8A**. Construct 10His-TAT-GFP is a control that lacks cytoxMLS and should not accumulate in mitochondria (Del Gaizo, and Payne, 2003). In constructs 10His-11ARG-cytoxMLS-GFP and 10His-11ARG-GFP, TAT is replaced by a run of 11 arginines, also known to constitute an efficient PTD (Mitchell, *et al*, 2000). The purified proteins are shown in

Figure 8B, both on a Coomassie stained gel (left panel) and on a Western Blot performed with an anti-His-tag antibody: lane 1, size marker; lane 2, 10XHis-11XArg-GFP; lane 3, 10XHis-11XArg-cytoxMLS-GFP; lane 4, 10XHis-TAT-GFP; lane 5, 10XHis-TAT-cytoxMLS-GFP. A doublet is seen for both 10XHis-11XArg-cytoxMLS-GFP and 10XHis-TAT-cytoxMLS-GFP that likely indicates C-terminal degradation of the protein. The cause of this degradation is unknown.

We have initiated confocal microscopy experiments aimed at following the cellular localization of 10His-TAT-cytoxMLS-GFP in live cells. Y-1 mt-DsRed mouse cells (see "Uptake of Isolated Mitochondria" section below for a cell line description) expressing mitochodrially localized DsRed protein (red in **Figure 8, C-E**), were incubated with 10His-TAT-cytoxMLS-GFP. Green fluorescence accumulates in the cells immediately after their incubation with the protein (compare **Figure 8 D**, cells treated with 10His-TAT-cytoxMLS-GFP, to **C**, untreated cell control. Note that not all the cells express mt-DsRed to the same extent. Magnification 10X). However, inspection of the cells at higher magnification (60X) shows that the two labels do not co-localize, indicating that the vast majority of the exogenous protein is trapped in a non-mitochondrial cellular compartment after its uptake by the cell. We presently, do not understand the reason for the discrepancy between our results and those of Del Gaizo & Payne (Del Gaizo, and Payne, 2003). As part of our ongoing collaboration with the Payne lab, we have sent them 10His-TAT-cytoxMLS-GFP so they can directly compare its activity to that of the original TAT-MLS-GFP with the mMDH MLS(Del Gaizo, and Payne, 2003). It is possible that 10His-TAT-cytoxMLS-GFP protein is trapped in endosomes after uptake and that it never reaches the mt-surface, as reported for some TAT peptides (Chauhan, *et al*, 2007). To avoid this possibility, we are starting a new series of experiments using the protein Lysteriolysin O (LLO), which mediates endosome escape during the life cycle of the intracellular pathogen *Lysteria monocytogenes* (Pizarro-Cerda, and Cossart, 2006), to release 10His-TAT-cytoxMLS-GFP into the cell's cytoplasm. In addition we are starting similar experiments with the constructs carrying an 11ARG PTD (**Figure 8A**).

The modification introduced by Khan and Bennett (Khan, and Bennett, 2004) to the method of Del Gaizo & Payne (Del Gaizo, and Payne, 2003) was to replace the GFP domain of TAT-MLS-GFP with a DNA binding protein. This resulted in a fusion protein that reportedly achieved efficient transformation of mammalian mitochondria. More specifically, they used the Mt-Transcription Factor A (TFAM), which functions both as a transcription factor and a histone in the matrix of mammalian mitochondria (Khan, and Bennett, 2004). A note of caution should be introduced here. While the claims behind the protofection technique are far reaching, no methods have been made available to the scientific community and, so far, the published results remain non replicated (Khan, and Bennett, 2004). For this reason, we have decided that it is for the best interest of the project, to spend time and effort testing the biochemical performance of all the protofection constructs that we create. Following the little detail given in Khan & Bennett (Khan, and Bennett, 2004), we have made the construct 10His-TAT-cytoxMLS-TFAM which fuses TFAM to TAT and cytoxMLS (see scheme in **Figure 9A**). **Figure 9B** shows a Coomassie stain of the purified protein (top panel) and Western Blots performed with anti-His-tag (bottom, left panel) and anti-TFAM antibodies (bottom, right panel). In addition, we have made other fusion TFAM constructs lacking either TAT, or cytoxMLS, or both, to determine the influence of these peptides on the activity of TFAM. All these constructs are shown in **Figure 9A**. We have tested the ability of construct 10His-TAT-cytoxMLS-TFAM to bind DNA by means of

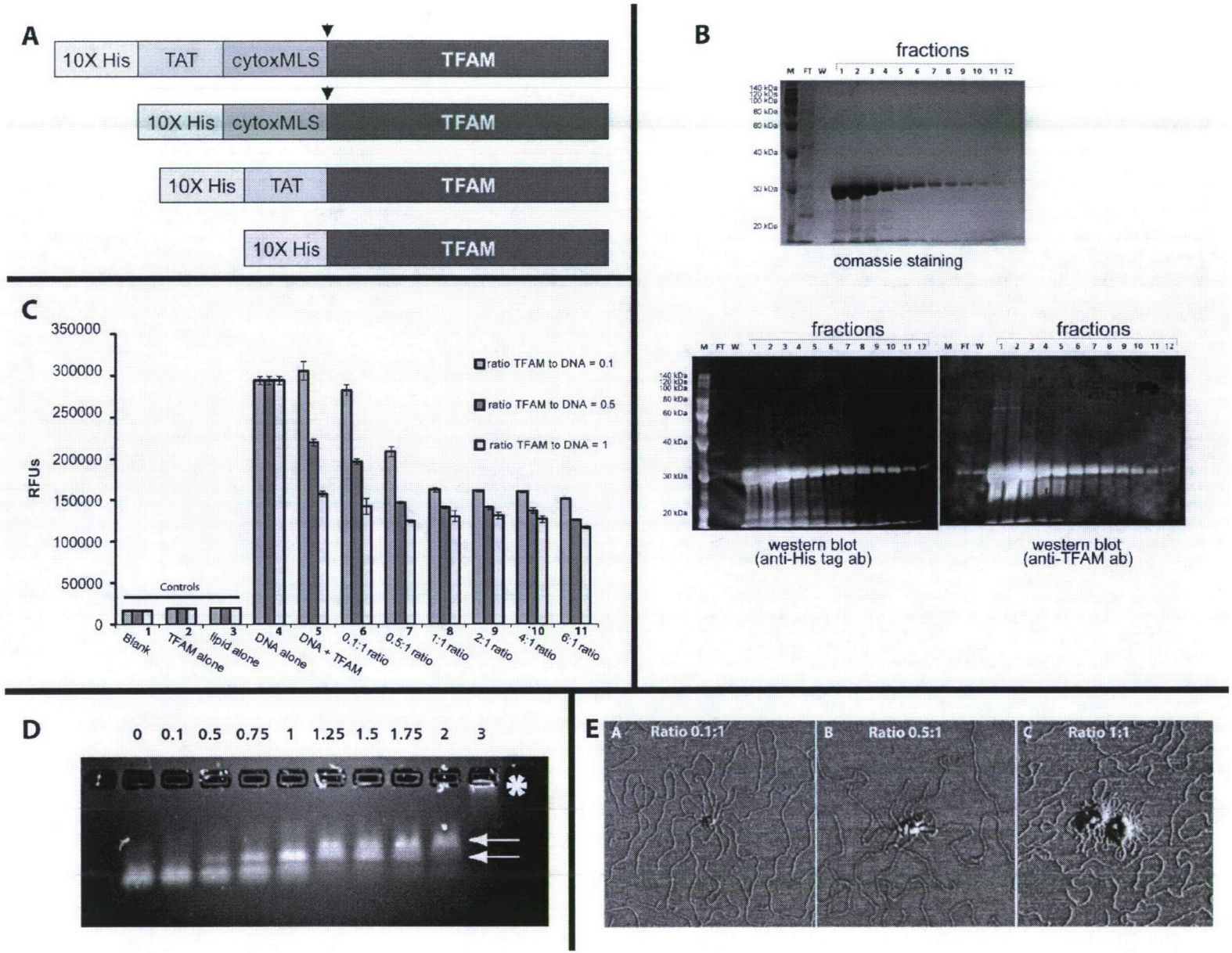

A
10X His
TAT
cytoxMLS
TFAM
10X His
cytoxMLS
TFAM
10X His
TAT
TFAM
10X His
TFAM
B
fractions
comassie staining
western blot
(anti-His tag ab)
western blot
(anti-TFAM ab)
C
RFUs
ratio TFAM to DNA = 0.1
ratio TFAM to DNA = 0.5
ratio TFAM to DNA = 1
Controls
Blank
TFAM alone
lipid alone
DNA alone
DNA + TFAM
0.1:1 ratio
0.5:1 ratio
1:1 ratio
2:1 ratio
4:1 ratio
6:1 ratio
D
0 0.1 0.5 0.75 1 1.25 1.5 1.75 2 3
E
A Ratio 0.1:1
B Ratio 0.5:1
C Ratio 1:1

Figure 9

Sybr assays, electrophoretic mobility shift assays (EMSA), and Atomic Force Microscopy (AFM) assays. Sybr assays measure the amount of fluorescence appearing as a result of Sybr Green's binding to DNA. We reasoned that TFAM binding could restrict Sybr Green's access to DNA and thus its DNA-induced fluorescence (Weissig, *et al*, 1998). The results of these experiments are shown in **Figure 9C** and clearly indicate that the addition of a 10His tag, TAT, and cytoxMLS does not abolish the DNA binding activity of TFAM. The figure shows a dramatic increase of fluorescence upon addition of a 4.8 kb plasmid (compare the **Control** lanes to lane **4**). The Sybr-Green fluorescence is decreased by pre-incubation of the DNA with increasing concentrations of TFAM (compare set of columns **4** to set of columns **5**) indicating that TFAM binding to the DNA prevents Sybr Green's access to it. Similarly, pre-incubation of the DNA with increasing concentrations of Fugene 6® liposomes (Roche) restricts the access of Sybr Green to DNA at all TFAM:DNA ratios (compare sets of columns **6-11** to sets of columns **4** and **5**; ratios of lipid to DNA, v/w, are indicated).

The results of the Sybr assays were reconfirmed by EMSA analysis. A small DNA duplex labeled with fluorescein was incubated with increasing concentrations of TFAM and the formation of a complex was monitored by electrophoresis. In addition to showing that the binding of TFAM alters the mobility of the DNA duplex (white arrows in **Figure 9D**), the binding assays also show that at high protein/DNA ratios the DNA is shifted into high molecular weight complexes that are trapped in the gel well (asterisk in **Figure 9D**). Since the formation of such complexes could potentially affect the mt-transforming activity of 10His-TAT-cytoxMLS-TFAM, we have further investigated the stoichiometry of its interaction with DNA by means of AFM techniques. AFM analysis confirmed our suspicion about the formation of high molecular weight complexes at higher 10His-TAT-cytoxMLS-TFAM:DNA ratios (compare **left**, **central**, and **right** panels in **Figure 9E**; TFAM:DNA ratios 0.1:1, 0.5:1, and 1:1 respectively). Also clear from the images is that 10His-TAT-cytoxMLS-TFAM does not coat the DNA molecules (**Figure 9E**). Instead AFM analysis suggests that the initial binding of the protein to the DNA nucleates the formation of a nucleoid-like structure, in agreement with the results of Kaufman *et al.* (Kaufman, *et al*, 2007). These results will be important for the design of the protofection assays to be performed *in vivo*. Presently, we are in the process of analyzing the DNA binding activity of all the other TFAM constructs of **Figure 9A** to determine the influence of all the different protein domains on the DNA binding activity of TFAM.

We have already tested the mt-transformation activity of 10His-TAT-cytoxMLS-TFAM in preliminary protofection assays. The results are very promising. **Figure 10**A shows the results of an experiment in which EPH4 cells labeled with CMX-Ros Mitotracker (Invitrogen) (red in panels **1** and **3**) were incubated with 10His-TAT-cytoxMLS-TFAM pre-complexed with an Alexa-488-labeled, synthetic, double-stranded, DNA oligonucleotide (green in panels **2** and **3**). Co-localization of the 2 dyes (yellow in panel **3**) suggests that the labeled DNA has entered the mt-compartment. Bright field view is shown in panel **5**.

DELIVERY OF DNA TO MITOCHODRIA BY TRANSFECTION REAGENTS AND VESICLES: Attempts to use liposomes to deliver DNA to mitochondria have been reported. In particular, DeQuAlinium based vesicles (DQAsomes) and liposomes with surface-linked triphenylphosphonium cations have been named the first available mitochondria-targeted nano drug and DNA delivery systems (Weissig, *et al*, 2006). These liposomes have been

reported to colocalize with mitochondria by confocal microscopy and to deliver DNA to the organelle (D'Souza, *et al*, 2005).

We have used three types of vesicles to test their performance in delivering DNA to

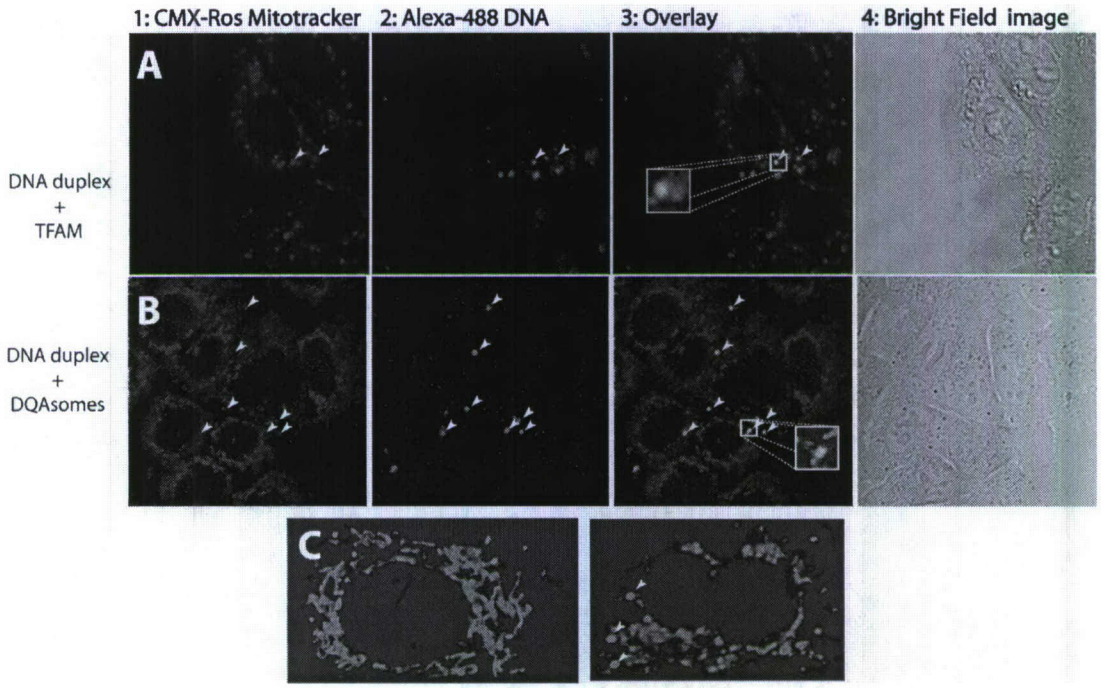


Figure 10

mitochondria, Lipofectamine 2000 (Invitrogen), Fugene 6® (Roche) and DQAsomes (generous gift from G.G. D'Souza). We have obtained the best results with the DQAsomes. **Figure 10**B shows that incubation of EPH4 cells with DQAsomes complexed with an Alexa-488-labeled, synthetic, double-stranded, DNA oligonucleotide (green in panels **2** and **3**), results in the localization of some of these complexes to the mitochondria (red in panels **1** and **3**). The amount of co-localized complexes appears to be much higher than with the protofection method (**Figure 10A**).

In addition to these compounds, Polyethylenimine (PEI) conjugated to an MLS has also been proposed as a vehicle to deliver DNA to the mitochondria (Lee, *et al*, 2007). We are planning on testing this system to attempt the delivery of mitochondrial specific constructs to the mt-matrix.

BIOLISTIC TRANSFORMATION: Delivery of mtDNA to the mitochondria by means of a biological ballistic (biolistic) gun has been achieved in yeast but it has thus far not been reported in mammalian cells (see the Yeast section below) (Khan, *et al*, 2007). There is no reason, in principle, that would impede the biolistic transformation of mammalian mitochondria. Indeed, the biolistic method has been employed for the nuclear transformation of tissue culture

cells (Heiser, 1994; Jiao, *et al*, 1993; Yang, *et al*, 1990). For all these reasons, we are currently attempting the biolistic transformation of mouse mitochondria *in vivo*. Initial attempts with the cytoplasmic reporter construct GFPmito6NM have confirmed the suitability of the method for the introduction of DNA in our cell lines (not shown). In order to increase the chances of reaching mitochondria with the biolistic projectiles, we have planned to increase mt-size by the use of chemicals known to induce mitochondrial swelling. This swelling process is well documented and results in the formation of megamitochondria (Karbowski, *et al*, 1997; Karbowski, *et al*, 1999; Matsuhashi, *et al*, 1996). **Figure 10C** shows a comparison of a normal EPH4-DsRed cell (**left** panel) and a cell that has been treated with 50μg/ml of CAM for 4 days (**right** panel). Note the swelling of the mitochondria (white arrows). Since this process is reversible (data not shown), we are currently using cells transiently treated with CAM in biolistic transformation assays.

ELECTROPORATION: Isolated mammalian mitochondria can be transformed by electroporation (Yoon, and Koob, 2003). Electroporation assays with these constructs and isolated mitochondria are currently being performed in the lab.

BACTERIAL CONJUGATION: A new method for delivery of DNA to mitochondria has been recently described that takes advantage of the species promiscuity of bacterial conjugation (Yoon, and Koob, 2005). A problem with this method is that it only transfers single stranded DNA. Whether this DNA can be used by the mt-DNA repair machinery to synthesize a double stranded DNA molecule is presently unknown. Nevertheless, we have decided to test this approach by adding an origin of transfer, OriT, to some of the reporter constructs of **Figure 8.** As mentioned above, we are testing first whether these constructs are capable of driving reporter expression in the mt-matrix by means of biolistic transformation and electroporation. OriT has also been already added to some of the genomic constructs shown in **Figure 5D**.

UPTAKE OF ISOLATED MITOCHONDRIA: The electroporation method of transformation requires that the isolated mitochondria be placed back into the host cell. Several techniques have been described to do this (Clark, and Shay, 1982; King, and Attardi, 1988; Nass, 1969), the most straight forward of which appears to simply require phagocytosis (Clark, and Shay, 1982). We initially attempted to use confocal microscopy to follow phagocytosis of red mitochondria tagged with CMX-Ros mitotracker by cultured mouse cells pre-labeled with FM-Mitotracker (green). Unfortunately, acute photobleaching of the mitotracker labels complicated the experiments. For this reason, we decided to create mouse cell lines that stably express mitochondrially localized DsRed. These cell lines were created in the lab by lipofectamine mediated stable transfection of the mammalian expression vectors pDsRed2-Mito (Clontech) and peGFP-mito, made by replacing the DsRed gene in pDsRed2-Mito with the GFP gene from pIRES2-eGFP (Clontech). PDsRed2-Mito and peGFP-mito encode fusions of either DsRed or GFP with the MLS of subunit VIII of human cytochrome C oxidase (see protofection section above) (Rizzuto, *et al*, 1989; Rizzuto, *et al*, 1995) and their transfection into EPH4 cells resulted in either red (EPH4-mito-dsRed, **Figure 11A**, cell nucleus in blue due to Hoechst staining) or green mt-networks (EPH4-mito-GFP, **Figure 11B**). Red mitochondria isolated from EPH4-mito-dsRFP cells were incubated with attached EPH4-mito-GFP cells and the fate of the red mitochondria was followed by confocal microscopy. The results show that EPH4 cells are capable of internalizing isolated mitochondria, see white arrows in **Figures 11C**, **11D**, **11E**, and

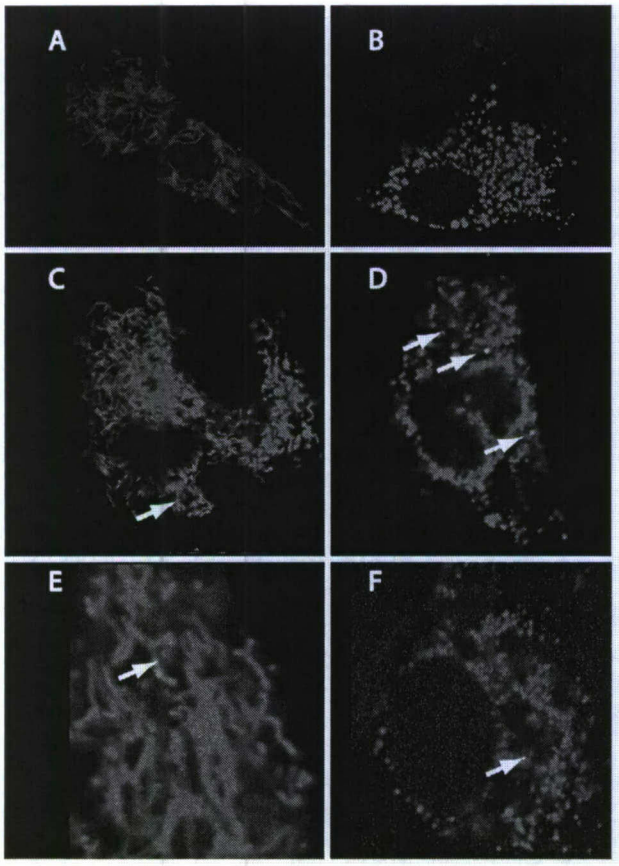


Figure 11

11F. However, whether internalized mitochondria fuse with the existing mt-network is still unclear. In fact, we have seen that, at least, part of the phagocytosed mitochondria end up in lysosomes, an indication that they are degraded by the cell. This is shown in **Figure 11F** by the co-localization of lysosomes labeled with Lysotracker Blue (Invitrogen) with red mitochondria (white arrow). We are performing more experiments aimed at elucidating the fate of internalized mitochondria after phagocytosis.

A much more powerful tool to test the ability of isolated mitochondria to fuse with the internal cellular mt-network upon phagocytosis is to use a genetic selection approach. One genetic marker that has been traditionally used in the study of mt-physiology is resistance to chloramphenicol (CAM). The scheme below (**Figure 12A**) shows the experimental outline based on the results of Clark & Shay (Clark, and Shay, 1982). These authors reported that CAM-sensitive (CAM^s) mouse culture cells (with mitochondria shown as green ovals) became resistant to the antibiotic after incubation with mitochondria isolated from CAM resistant (CAM^r) cells (with mitochondria shown as red ovals), followed by selection in the presence of CAM. We have obtained from the laboratory of Dr. D. Wallace a cell line (501-1) that is CAM^r due to a homoplasmic T-C transition at position 2433 of the mt-16S rRNA gene (Blanc, *et al*, 1981; Bunn, *et al*, 1974). We also have the CAM^s cell line Y-1 (ATCC # CCL-79) reported by Clark *et al*. (Clark, and Shay, 1982) to become CAM^r upon internalization of CAM^r, purified mitochondria via phagocytosis. To increase the selective pressure in favor of the exogenous CAM^r mitochondria (King, and Attardi, 1988), the recipient, CAM^s cells were devoid of their mt-DNA, i.e. they were made rho^0 (ρ^0) as follows.

Creation of ρ^0 Mitochondria: Various techniques for the creation of ρ^0 cells have been described, including treatment with ethidium bromide (EtBr) (King, and Attardi, 1989) and silencing of the mt-DNA polymerase by RNAi (Khan, and Bennett, 2004). **Figure 12B** shows the results of treatment of EPH4 cells with varying concentrations of EtBr (horizontal axis. Time of incubation with EtBr is also indicated). The percentage of remaining mtDNA, relative to an untreated control, was determined by Real-Time PCR with a Bio-Rad IQ5 apparatus (vertical axis). In our hands, treatment of these cells with 2μg/ml EtBr for at least 2 days reduces the amount of mtDNA to less than 10% of its original amount **Figure 12B**.

Preliminary experiments aimed to reproduce the results of Clark & Shay (Clark, and Shay, 1982) showed that CAM^s cells incubated with CAM^r mitochondria become more resistant

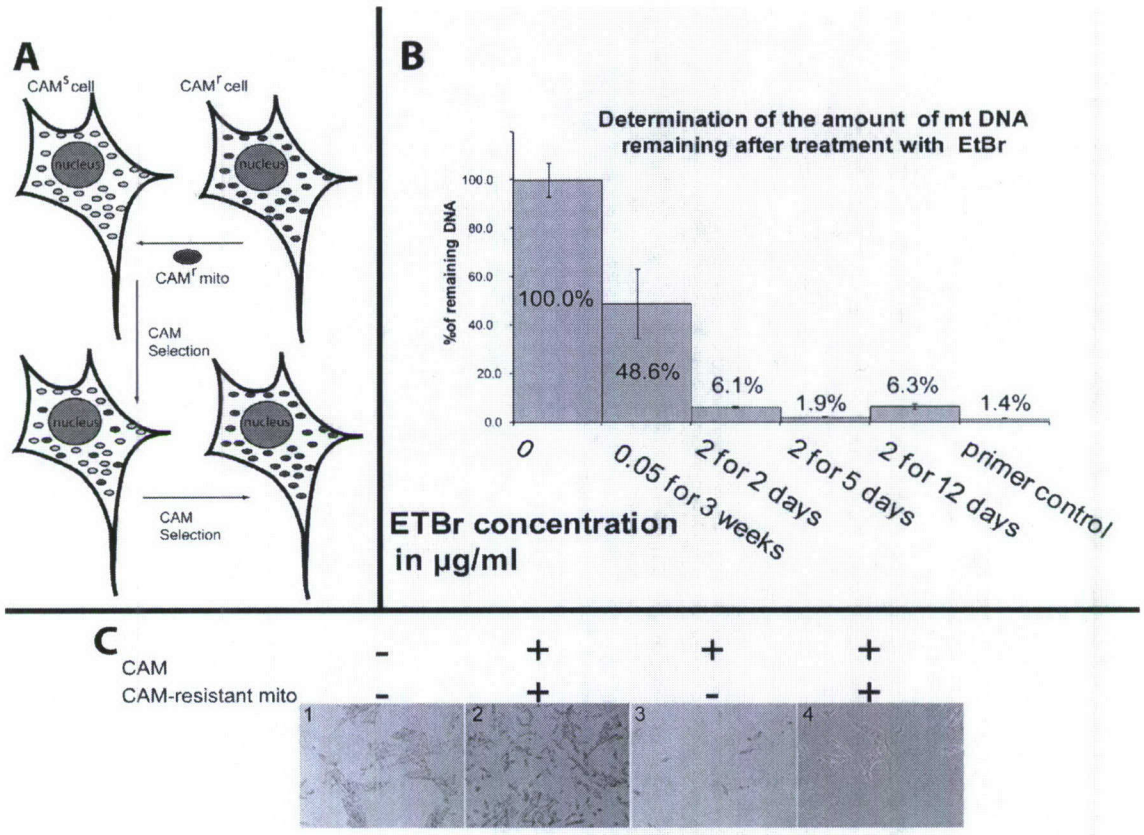


Figure 12

to the antibiotic than control cells (**Figure 12C**, compare panel **1** to panel **3**). Cells that received CAM^r mitochondria could be detached from the plate by trypsin treatment and replated, giving rise to small colonies (**Figure 12C**, panel **4**). However, we also observed that the viability of these colonies ceased after two weeks following selection. On the contrary, Clark & Shay (Clark, and Shay, 1982) have reported that CAM^r clones arising from their selection screens were phenotipically stable for at least 12 weeks. While we currently cannot explain the discrepancies between our results and those of Clark & Shay (Clark, and Shay, 1982), there exist several reasons known to complicate the outcome of these experiments. For example, it has been known for long that the expression of certain mitochondrial deficient phenotypes, such as rho^0 and CAM^r requires the absence of pyruvate and uridine from the medium (King, 1996 and references therein). This is exemplified by the results of **Figure 13**, showing a CAM titration experiment performed in the presence of two different media formulations: **A**) DMEM High Glucose, 50% F-12, 50% Mix. (UCSF Cell Culture Facility cat. # UCSFCCFAA010), supplemented by manufacturer with 3.15 g/L Glucose, 0.055 g/L Na-Pyruvate, 0,37 g/L L-Glutamine, 2.44 g/L $NaHCO_3$, and by us with 2.5% Fetal Bovine Serum (FBS) and 15% Horse Serum (HR) and **B**) DMEM from JR Scientific Inc. lacking pyruvate (cat. # 30039), supplemented with 2.5% FBS and 15% HR. We failed to detect any CAM^s of EPH4 and Y-1 cells with the former formulation even at CAM concentrations of 200 µg/ml (**Figure 13A**, cell type and CAM concentration is shown on the left, time points are shown above panels), much higher than the 50 µg/ml, normally used in similar experiments (Clark, and Shay, 1982). In contrast, when we switched to the formulation lacking pyruvate we were able to detect CAM^s even at CAM concentrations of 50

μg/ml (**Figure 13B**). Y-1 cells are clearly more sensitive to CAM than EPH4 cells, as they die faster and at lower concentrations than the latter (compare in top right panel to middle right panel in **Figure 13B**). We initially attributed the appearance of CAM^s solely to the absence of pyruvate in the formulation from from JR Scientific Inc. The reasons behind the appearance/absence of this phenotype have to be more complicated however, as both Clark & Shay (Clark, and Shay, 1982) and us have been able to see CAM^s in the presence of pyruvate; i.e. the medium used in the experiments of **Figure 12C** was DMEM Low Glucose from UCSF Cell Culture Facility (cat. #UCSFCCFAA001), supplemented by manufacturer with 1.0 g/L Glucose, 0.11 g/LNa-Pyruvate, 0,584 g/L L-Glutamine, 3.7 g/L $NaHCO_3$, and by us with 10% Fetal Bovine Serum (FBS), whereas Clark & Shay (Clark, and Shay, 1982) describe their medium as Dulbecco's modified medium with 1 g/L glucose, pyruvate (actual concentration not reported) and 10% FBS. Therefore, other components in the formulation must also affect the appearance of a CAM^s phenotype. The experiments of **Figure 13** are important, however, because they establish appropriate selective conditions that can be used for the repopulation of CAM^s cells with CAM^r mitochondria. We are currently in the process of repeating the experiments of **Figure 12C** in medium with higher selective power, i.e. the medium used in **Figure 13B** with higher concentrations of CAM. We are also in the process of analyzing what components of the medium are critical for the expression of the CAM^s phenotype.

MICROINJECTION: This technique has been succesfully used to transfer CAM-resistant mitochondria to ρ^0 cells (King, and Attardi, 1988). While we would prefer to use one of the above techniques to transform mitochondria, we are not ruling out the use of microinjection. To facilitate the microinjection procedure and to follow the fate of exogenous mitochondria by microscopy, we have constructed 501-1 cell lines with fluorescently tagged mitochondria by the expression of mitochondrially targeted GFP and DsRed proteins as described in the section entitled "Uptake of Isolated Mitochondria" above).

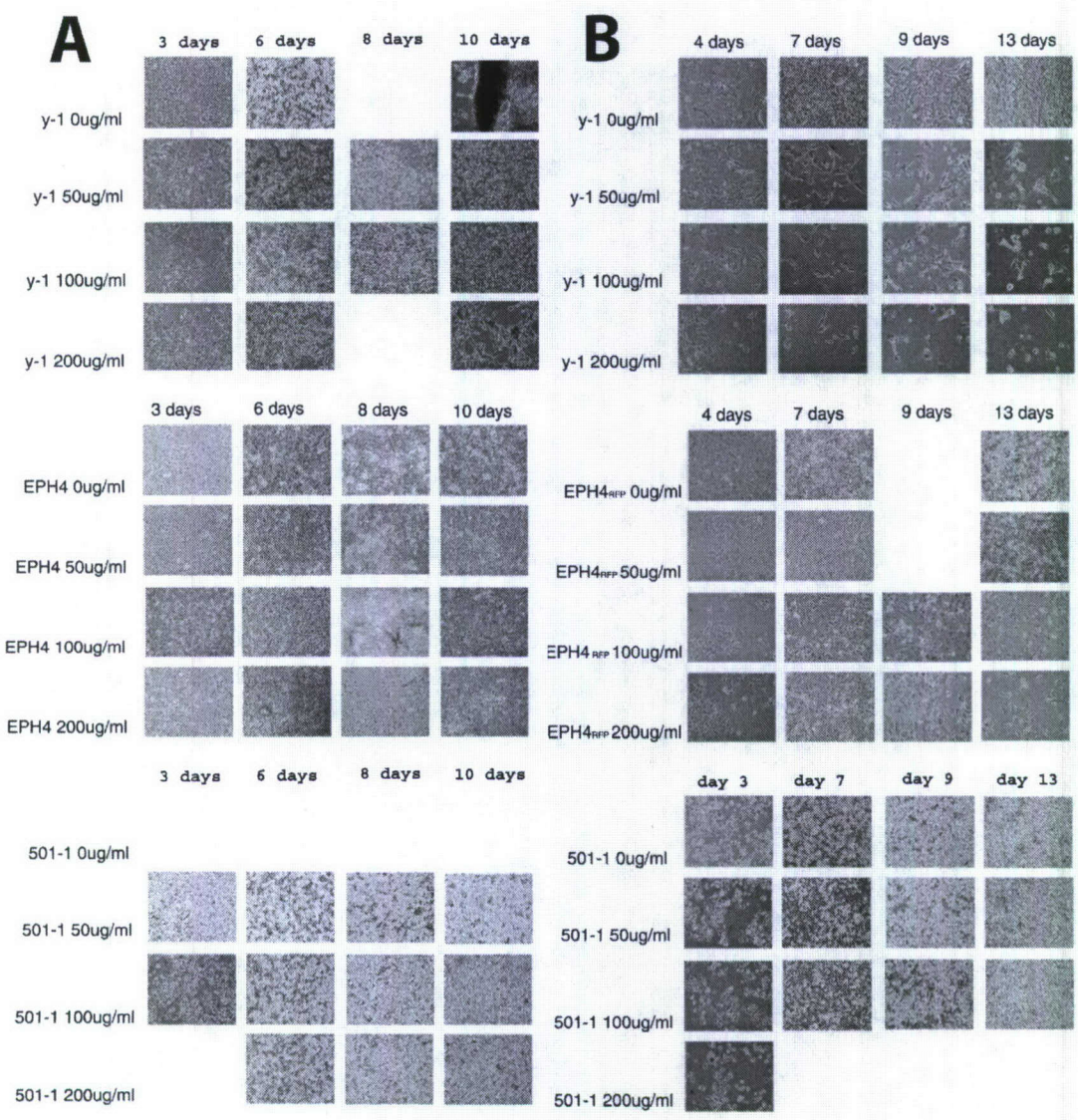

A
3 days
6 days
8 days
10 days
y-1 0ug/ml
y-1 50ug/ml
y-1 100ug/ml
y-1 200ug/ml
3 days
6 days
8 days
10 days
EPH4 0ug/ml
EPH4 50ug/ml
EPH4 100ug/ml
EPH4 200ug/ml
3 days
6 days
8 days
10 days
501-1 0ug/ml
501-1 50ug/ml
501-1 100ug/ml
501-1 200ug/ml
B
4 days
7 days
9 days
13 days
y-1 0ug/ml
y-1 50ug/ml
y-1 100ug/ml
y-1 200ug/ml
4 days
7 days
9 days
13 days
EPH4RFP 0ug/ml
EPH4RFP 50ug/ml
EPH4 RFP 100ug/ml
EPH4RFP 200ug/ml
day 3
day 7
day 9
day 13
501-1 0ug/ml
501-1 50ug/ml
501-1 100ug/ml
501-1 200ug/ml

Figure 13

Specific Aim 2: Creation of a transcriptionally independent yeast mitochondrion.

Given the yet-unresolved difficulties encountered while attempting to deliver DNA to the mitochondria of mammalian cells, we have also included yeast mitochondria within our experimental scope. The reasons for doing this are the following: 1) protocols to deliver DNA to yeast mitochondria by means of "microprojectile bombardment" or "biolistic transformation" are well-established, 2) the yeast mt-genome contains large non-coding regions where exogenous genes can be inserted, and 3) the appropriate knockout strains for the genes of interest already exist or can be easily created.

Since our laboratory is new to yeast techniques, the project has basically proceeded on two fronts: 1) familiarization with and optimization of the "biolistic" procedure of mt-transformation and 2) design and construction of the strains and plasmids necessary to build a transcriptionally independent mitochondrion. More recently, we have added a third front in which we attempt to force the translational machinery present in yeast mitochondria to work in a manner that allows straight forward regulation of the flow of genetic information within the organelle.

FRONT 1: Mt-transformation: Familiarization and optimization.

The yeast mt-transformation protocol is a difficult and highly inefficient technique, requiring many steps. For this reason, we have initially attempted to recreate a published bombardment experiment. To carry out these experiments, we have obtained experimental advice from two leaders in the field, Thomas D. Fox at Cornell University and Golik Pawel at Warsaw University, Poland.

Figure 14 shows a schematic outline of the strategy followed to insert exogenous genes in yeast mitochondria, together with actual experimental results obtained in our lab. Briefly, a nuclearly encoded and mitochondrially recoded ARG8 gene, needed for arginine synthesis, is inserted within the COX3 coding region of the yeast mt-genome (Steele, *et al*, 1996). This series of experiments starts by the biolistic bombardment of strain DFS160 (leu2Δ arg8Δ, [ρ^0]) with a mixture of plasmids, a nuclear construct carrying a Leu2 nuclear marker and a mt-construct carrying a mitochondrially recoded ARG8M gene (**Figure 14A**, **left** panel) (Steele, *et al*, 1996). The ARG8M gene is placed between the flanking sequences of the mt-gene COX3, thus allowing its integration into the mt-genome in place of wt COX3 (**Figure 14A**, **left** panel). Since the efficiency of mt-transformation is low, initial selection for nuclear markers carried on the nuclear plasmid is essential. The **right** panel in **Figure 14A** shows the initial selection for cells expressing Leu2 in media lacking leucine. No growth was observed in a non-bombardment control run in parallel (data not shown). The **left** panel in **Figure 14B** shows a scheme of the mating step required for the identification of transformants carrying the mt-construct. The tester strain, GW22 ([ρ+ cox3-421]), carries a deletion mutation in the COX3 5'-untranslated leader that makes it non-respiring. The mating plate, obtained by replica plating the transformants onto a lawn of the tester strain grown on rich media, is shown in the right panel of **Figure 14B.** Clearly, the yield of mt-transformants is much lower than that of nuclear transformants (compare the number of colonies in **Figure 14A**, **right** panel to that of **Figure 14B**, **right** panel). Marker

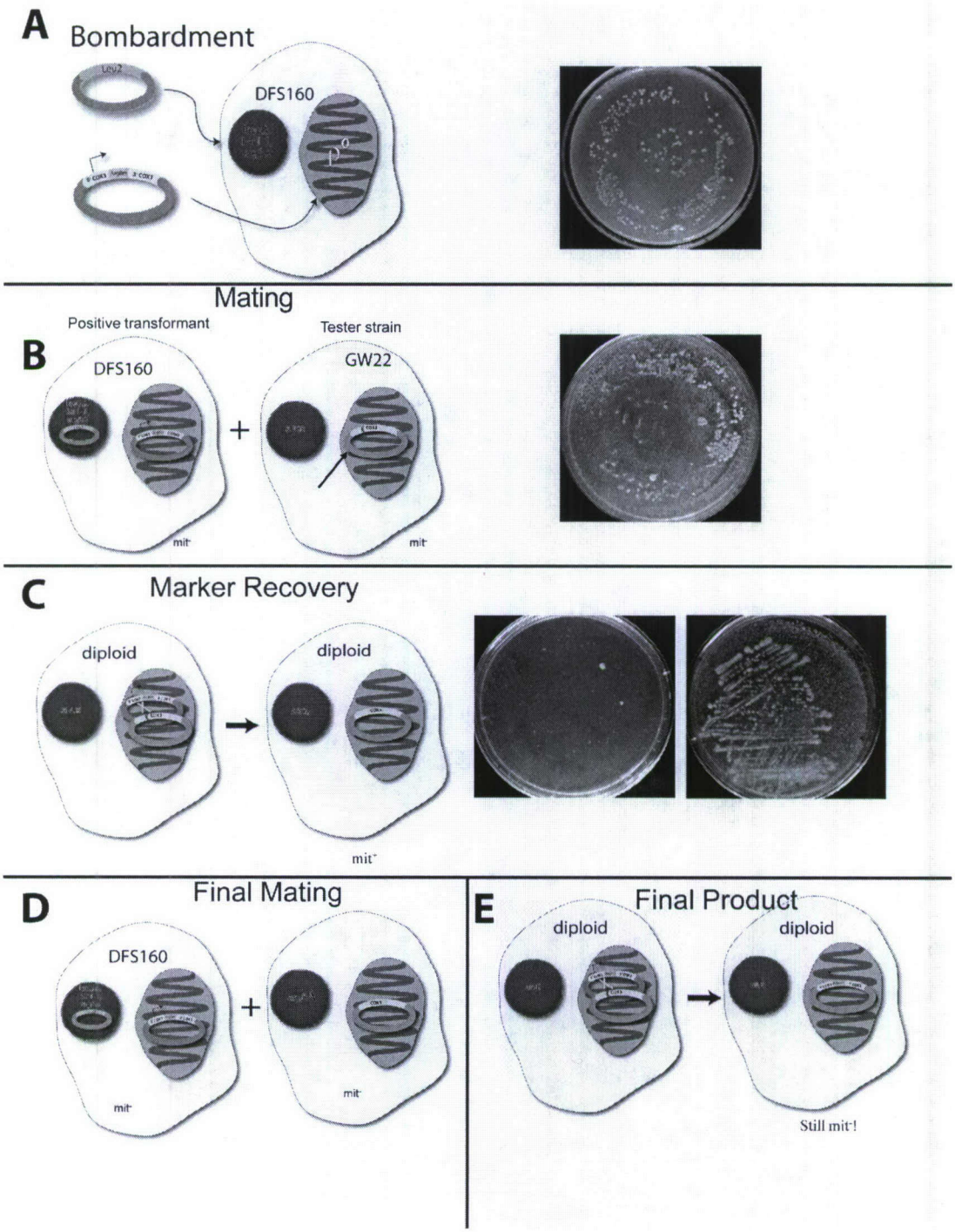


Figure 14

Recovery: Since the mutation in the tester strain can be complemented by the 5' region of COX3 flanking the ARG8 gene of the mt-construct, original transformants that have received this construct in their mt-matrix can be identified after mating with the tester strain. This requires a

homologous recombination event between the mt-genome of the tester strain and the mt-construct in the diploid cell obtained after mating, as shown schematically in **Figure 14C**. To select for respiring diploids, the mating plate is replica plated onto YPEG medium containing only non-fermentable carbon sources (**left** plate in **Figure 14C**). The original mt-transformants identified in **C** are purified away from non mt-transformats by several rounds of colony purification (right plate in **Figure 14C**), followed by marker recovery steps. Since we were confident at this stage that we had obtained pure mt-transformants, we proceeded to use this technique for the experiment described below, aimed at integrating the gene of the mt-RNA polymerase RPO41 into the mt-genome of yeast. For the sake of clarity, the final steps of the experiment of **Figure 14** will be explained here. First, the purified mt-transformants would be mated with a strain that allows the expression of the new mt-phenotype, i.e a strain with an ARG8Δ nuclear background, **Figure 14D**. Following homologous recombination, the mt-recoded ARG8 gene is inserted in place of COX3, resulting in a non-respiring diploid that is able to grow in the absence of arginine (**Figure 14F**).

FRONT II: Design and construction of a transcriptionally independent yeast mitochondrion.

In order to create a transcriptionally independent yeast mitochondrion, two genes need to be introduced in the mt-genome, the gene for the yeast mt-RNA polymerase, RPO41 and the gene for the Mt-Transcription Factor 1 MTF1. We have decided to start out with the RPO41 gene. Together with GenScript Inc., we have designed a synthetic version of the RPO41 gene that is recoded to comply with the yeast mt-genetic code and codon optimized to the mt-codon usage. The graph of **Figure 15A** compares the codon abundance of the nuclear copy of RPO41 to that of the synthetic RPO41, relative to mt-codon usage. Clearly, the codon composition of the synthetic sequence accommodates much better to the yeast mt-usage. Briefly, the synthetic construct, pRPO41m 4-4, consists of a codon optimized, synthetic RPO41 gene flanked by the 5' and 3' untranslated regions of the mt-gene COX2 (**Figure 15B**). In addition to the synthetic RPO41 gene, the construct carries an intact copy of the COX2 gene, so that homologous recombination between the construct and a mt-genome carrying a deletion in the 5' region of COX2 (ρ+ cox2-62), as shown in **Figure 15A**, will result in the integration of the synthetic gene upstream of COX2. This strategy will ensure that a functional copy of COX2 is left in the mt-genome, thus preserving mt-function in the synthetic cells (Mireau, *et al*, 2003). Construct pUC57-RPO41m, carrying the synthetic version of RPO41, flanked by COX2 sites, was created by GenScript Inc. (**Figure 15C**, lanes **4**, **9**, and **14**). Given the high bias towards AT rich sequences in the yeast mitochondrial genome, thus complicating the construction of synthetic mt-genes, building our synthetic RPO41 gene by GenScript Inc. lasted more than 4 months. pUC57-RPO41m was used to make a construct that could be used for the bombardment as follows. EcoRI digest of pUC57-RPO41m releases a fragment with the sequence of interest (black arrow in **Figure 15C**, lane **9**) that can be used to ligate into the large EcoRI-EcoRI fragment of the mitochondrial transformation vector pHM102 (white arrow in **Figure 15C**, lane **10**) (Mireau, *et al*, 2003). The resulting vector, pRPO41m 4-4, combines both fragments (black and white arrows in **Figure 15C**, lane **7**) and can be used to integrate the synthetic RPO41 gene into the mt-genome.

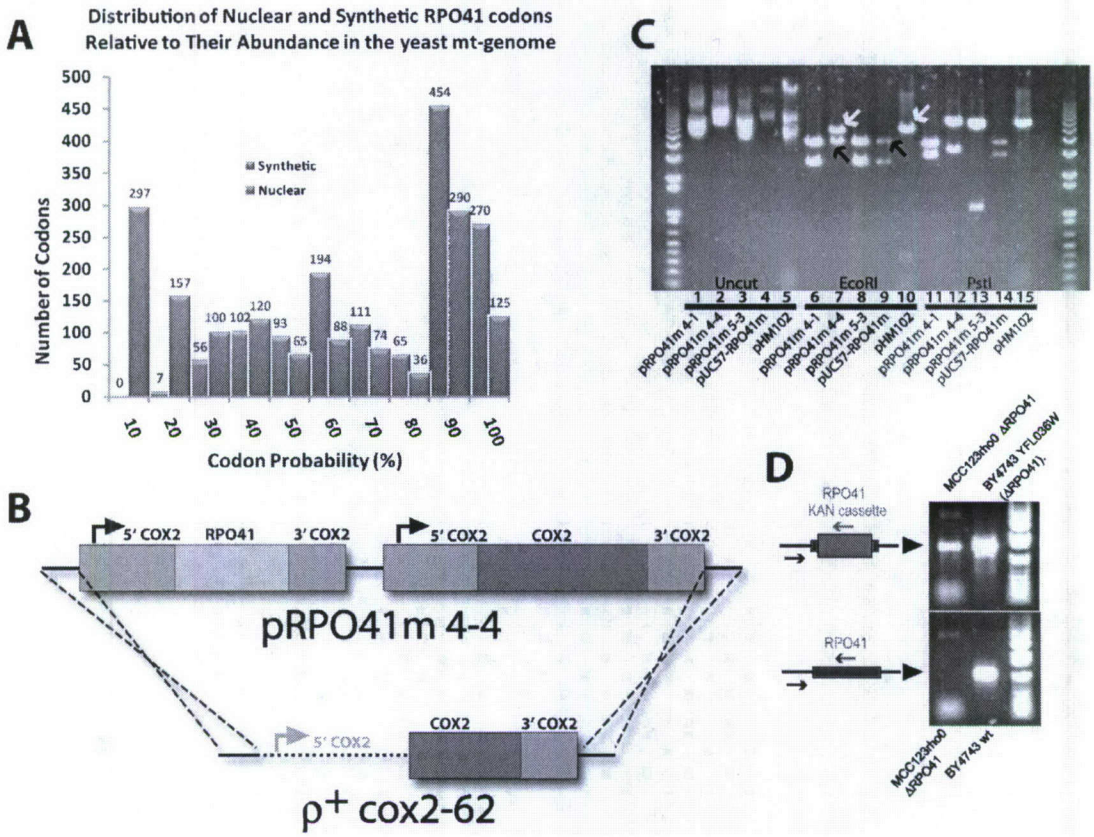


Figure 15

As a recipient for construct pRPO41m 4-4 we have made a derivative of the ρ^0 strain MCC123rho0 with a deletion of the nuclear copy of RPO41. MCC123rho0 is itself a derivative of strain DBY947, known to display a high efficiency of biolistic transformation (Bonnefoy, and Fox, 2002). To delete the RPO41 gene in MCC123rho0, strain BY4743 YFL036W, carrying a kanamycin (kanMX4) cassette replacing the RPO41 gene, was used to PCR amplify kanMX4 with the RPO41 flanking regions. KanMX4 confers resistance to the aminoglycoside antibiotic G418. The fragment was subsequently used to insert the kanMX4 cassette within the RPO41 gene of MCC123rho0, after standard lithium acetate transformation followed by selection in G418. We confirmed that G418 resistance was associated to RPO41 deletion in the resulting strain, BMZ1-1 (ade2 ura3 kar1-1 rpo41::KanMX4 [ρ^0]), by performing PCR analysis. The **top** panel in **Figure 15D** shows the PCR amplification of the RPO41 region with a primer outside of the RPO41 gene and a primer within the kanMX4 cassette. The presence of a PCR product after amplification of DNA from BMZ1-1 (lane **1**), with the same mobility as that resulting from BY4743 YFL036W (ΔRPO41) (lane **2**), confirms the replacement of the RPO41 gene of the former with the kanMX4 cassette. In the **bottom** panel of **Figure 15D** the RPO41 region was amplified with a primer outside of the RPO41 gene and a primer within the RPO41 gene. The absence of a PCR product after amplification of MCC123rho0 ΔRPO41, shows that most of the RPO41 coding sequence is deleted in this strain. A similar approach is currently being used to build strain BMZ3-2 (leu2-3,112 lys2 ura3-52 arg8::hisG rpo41::KanMX4 [ρ+ cox2-62] [pJJ1148 (RPO41, URA3 shuffle vector)]) a derivative of NB40-16D (Mireau, *et al*, 2003),

which will be used in the final step of transformation with a synthetic RPO41. BMZ3-2 will carry plasmid pJJ1148 (Cliften, *et al*, 2000), which will serve as a source of RPO41 protein until the synthetic gene is inserted in the mitochondria.

Construction of a yeast strain carrying a mitochondrial version of RPO41 has started in our laboratory. After initial bombardment of 16 plates containing BMZ1-1 lawns (**Figure 16A**, only 1 such plate shown), with a mixture of plasmids pRS316 (carrying a selectable URA3 gene) and pRPO41m 4-4, transformant colonies were selected in medium lacking uracil. Identification of mitochondrial transformants was performed by replica plating the plates of **A** onto lawns containing the tester strain HMD7 (lys2 [ρ+, cox2-107]), carrying a small deletion on the leader region of COX2 (**Figure 16B**) (Mireau, *et al*, 2003). Marker rescue identified two potential mt-transformants by their ability to grow on non-fermentable medium after mating (arrows in **Figure 16C**). These transformants will be colony purified and mated to strain BMZ-3 to select for the integration of RPO41 in the mt-genome.

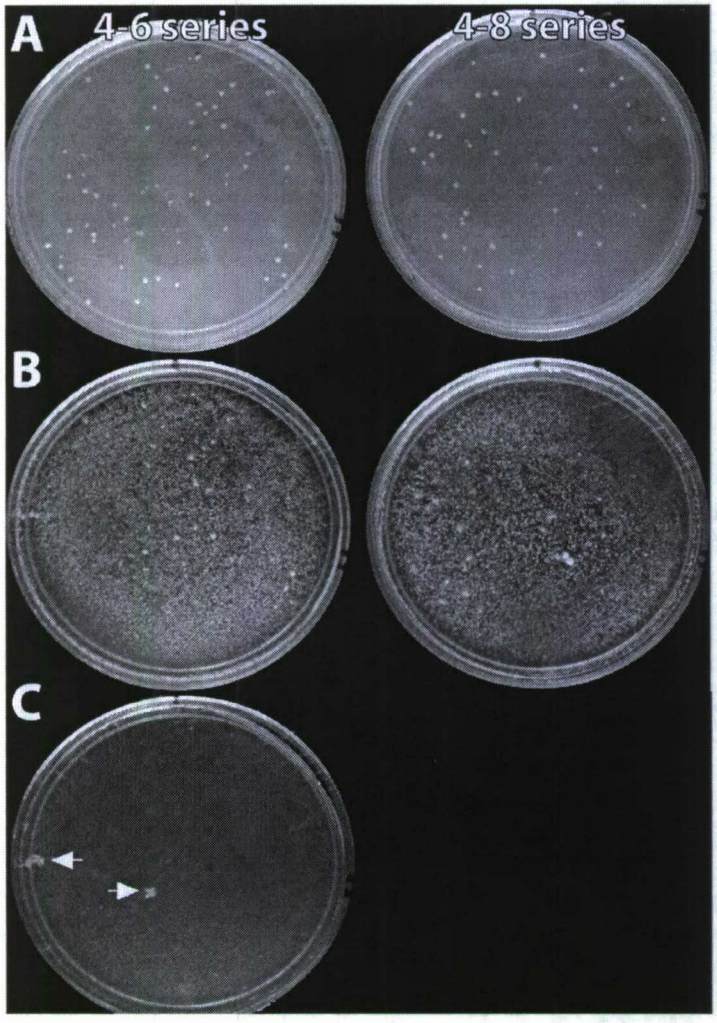


Figure 16

FRONT 3: Harnessing the mitochondrial ribosome for the establishment of straight forward regulatory systems in yeast mitochondria.

Insertion of genes into the yeast mt-genome has been achieved by flanking the gene of interest with 5' and 3' sequences of mt-genes (Amiott, and Jaehning, 2006; Bonnefoy, and Fox, 2002; Golik, *et al*, 2003; Steele, *et al*, 1996). While this strategy is a perfectly viable one to insert a reduced number of genes, the use of these flanking sequences will likely be a source of genomic instability as the number of exogenous genes increases. Moreover, one of our goals is to be able to regulate the expression of these exogenous genes. Regulation of gene expression in simple bacterial cells occurs mostly at the levels of transcription and translation. In particular, promoter strength during transcription and the affinity of the ribosome for the mRNA during translation initiation are well known processes that determine gene expression levels. Yeast mitochondria do not use either of these

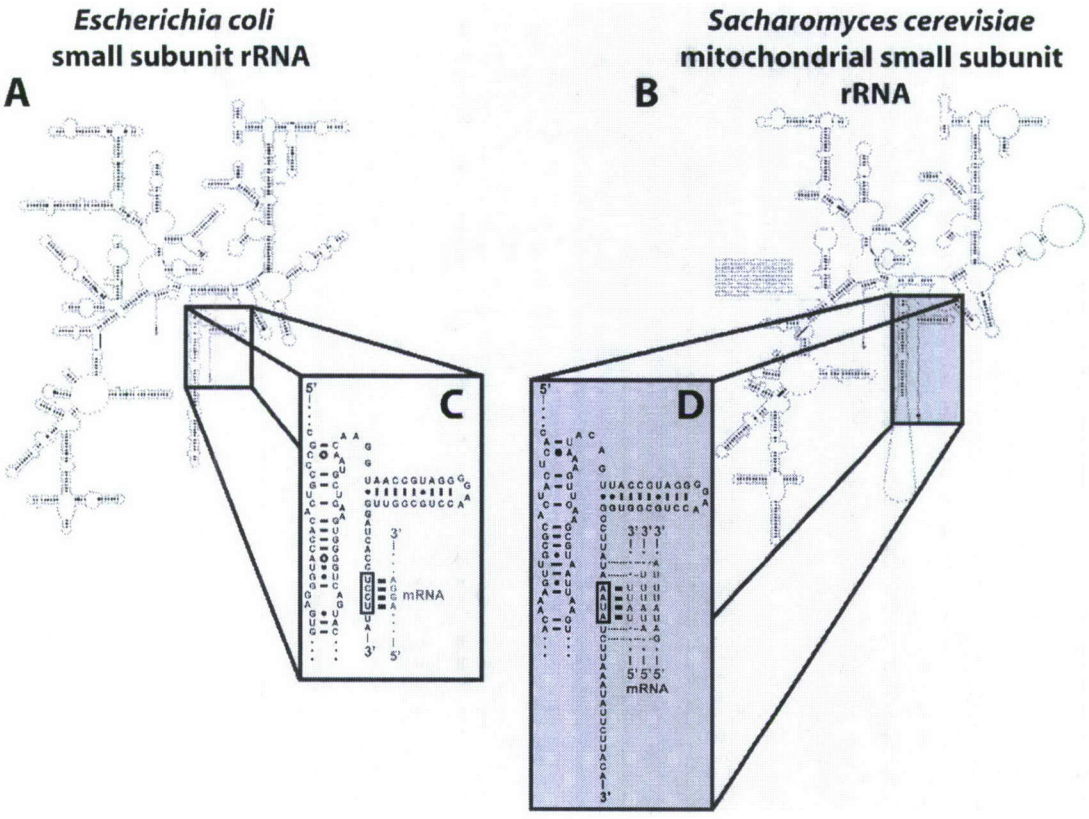


Figure 17

methods, as 1) the mRNA levels in mitochondria are not limiting, and 2) mitochondrial ribosome does not directly recognize the mRNA during initiation of translation and use instead a subset of proteins that work as translational activators (Costanzo, and Fox, 1988; Dunstan, *et al*, 1997; Mittelmeier, and Dieckmann, 1995). This is in contrast to the situation in bacteria, where a short sequence a few bases upstream of the initiation codon, the Shine-Dalgarno (SD) sequence, is used to target mRNAs to the ribosome during translation initiation (Jacob, *et al*, 1987; Shine, and Dalgarno, 1974). This occurs by the binding of the SD to its complementary sequence, the anti-Shine-Dalgarno sequence (aSD), located near the 3' end of the Small SUbunit (SSU) rRNA. Given that the structure of the functional core of the ribosome is highly conserved (Mears, *et al*, 2002), the possibility exists that yeast mitochondrial ribosomes could directly recognize mRNA features during translation initiation in a similar fashion as their bacterial counterparts. Use of alternative sequences at the aSD position to direct *E. coli* ribosomes to mRNAs that they would normally not be recognize has been performed in *E. coli* (Brink, and de Boer, 1998). Moreover, the concept has recently been improved to build networks of orthologous ribosome-mRNAs in the same cell (Rackham, and Chin, 2005). Our idea is to expand this concept to yeast mt-translation to allow the ribosome to recognize mRNA features in the absence of translational activators. Should we achieve this goal, a more direct relationship between mRNA levels and the final protein product would be obtained. This would endow us with powerful tools to control gene expression in the mt-matrix.

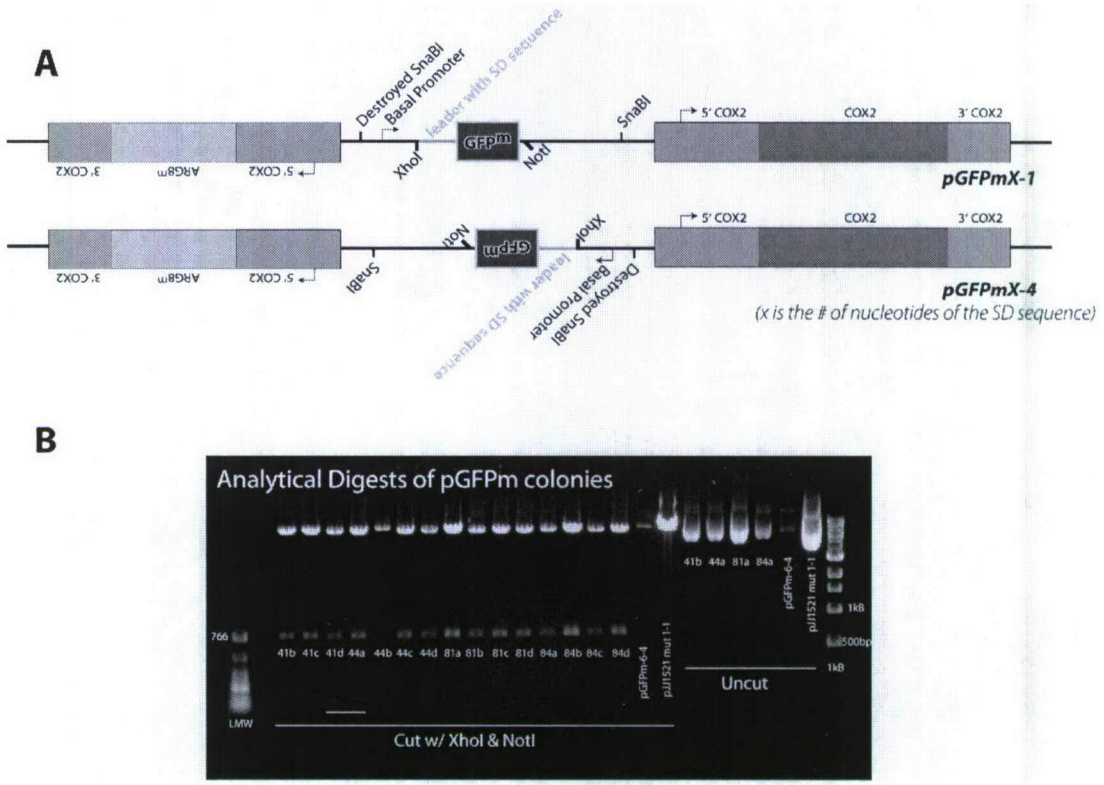


Figure 18

Figures 17A and **B** illustrate the high similarity between bacterial and yeast mt-ribosomes by comparing the secondary structure diagrams of the SSU rRNAs of *E. coli* and *Sacharomyces cerevisiae* mitochondria. **Figure 17C** shows the region near the 3' end of *E. coli* SSU rRNA bound to a schematic mRNA (SD sequence in red and aSD sequence boxed). **Figure 17D** shows the equivalent region in the SSU rRNA of yeast mitochondria bound to a hypothetical mRNA (SD sequence in red and aSD sequence boxed). We are currently designing some yeast mitochondrial constructs, which are designed to work as bacterial mRNAs and that will be placed in the mitochondrial genome by biolistic transformation. The idea is schematically shown in **Figure 17D**. Initially, we would create a set of GFP constructs that would place mt-SD sequences of different lengths upstream start codon in the absence of any yeast mt-leader regions, as these are known to direct the binding of translational activators to the mRNA (Costanzo, and Fox, 1988; Dunstan, *et al*, 1997; Mittelmeier, and Dieckmann, 1995). The size and composition of the linker between SD and start codon, 4 A residues, has been determined based on well-known *E. coli* mRNAs (Yusupova, *et al*, 2006). We have started the construction of the plasmids for biolistic transformation shown in **Figure 18A**. A GFP gene that can be expressed from the yeast mt-genome (y-mt-GFP) was obtained from the Fox lab (Cohen, and Fox, 2001). A PCR-amplified copy of this gene carrying the synthetic leader region was inserted between the ARG8-COX2 transcriptional unit and the COX2 gene present in plasmid pJJ1521 (**Figure 18B**) (Amiott, and Jaehning, 2006). Recombination between the region upstream of ARG8-COX2 and downstream of COX2 and the mt-genome will result in the integration of this gene in the mt-genome. **Figure 18B** shows the restriction analysis of transformants arising after

the final ligation of y-mt-GFP into a modified version of pJJ1521. Most of the constructs show the expected XhoI-NotI fragment, whose identity has been confirmed by sequencing analysis. Transformation of these constructs into the yeast m-genome will be performed soon.

It is possible that the sequence present at the aSD site in yeast mt-ribosomes is not optimal for targeting mRNAs to the ribosome during translation initiation. For this reason we are also planning on mutagenizing the mt-ribosomes themselves to create a subset of orthologous ribosomes in the organelle that would be targeted to their cognate mRNAS (Rackham, and Chin, 2005).

Future Prospects

Future tasks will involve the incorporation of the basic functions needed to sustain independent life of the mitochondrion. Throughout this process we hope to learn the genomic designs that are consistent with a minimal cell complexity. The accomplishment of the final goal, i.e. the creation of a minimal cell, will require many steps similar to the ones described above. We cannot at present give an exhaustive list of all the genes whose transfer to the mt-genome will be required for the successful construction of this cell. However, we can delineate our long-term strategy as follows. It is not our goal to attempt a reconstruction of the original alpha-protobacterium that gave rise to modern mitochondria. Instead, we will attempt to build the simplest cell possible using modern mitochondria as the platform. We foresee the use of heterologous genes of diverse origins whenever their insertion in the new mt-genome would result in a simpler design, circunvent technical difficulties, or shorten the time required for construction of the new cell. For example, in Chinese hamster and human cells the 43-subunit NADH-quinone oxidoreductase, can be replaced with the single-subunit NADH-Q oxidoreductase of *S. cerevisiae* mitochondria (Seo, *et al*, 1998; Seo, *et al*, 2000). Clearly, being able to use the yeast enzyme in the context of the mouse mitochondria would spare the need to insert and knock out 42 peptides.

Other stages of the project will certainly include:

- Addition of the genes involved in mRNA processing.
- Addition of the genes necessary to build an energetically independent mitochondrion.
- Addition of the genes involved in synthesis and maintenance of membranes.
- Creation of a translationally independent mitochondrion.
- Addition of the genes necessary for mt-division.
- Creation of a minimal stress response.

Complications that are expected to arise may include:

- Genome size will rapidly increase. This could hamper the use of *E. coli* as a platform to engineer synthetic mt-genomic constructs and/or the delivery of these constructs to the mouse mitochondria. This may eventually lead us to resort to homologous recombination as a way to insert additionals genes into the mt-genome. Homologous recombination seems to be present in mammalian

mitochondria (Thyagarajan, *et al*, 1996), although this is a highly controversial matter (Sato, *et al*, 2005 and references therein). We are also planning on modifying the lambda Red, recombineering system (Yu, *et al*, 2000) to facilitate recombination in mitochondria.

- Regulation of the expression of a large number of exogenous genes in the new genome. As mentioned above, a first approach towards solving this problem will be taken as part of the initial steps described here for the mouse mitochondria. However, it is clear that regulatory strategies more complex than the simple use of promoter strength will have to be devised.

  As for yeast mitochondria, an initial obstacle towards being able of regulating gene expression in the organelle has been described above (see the section "Harnessing the mitochondrial ribosome for the establishment of straight forward regulatory systems in yeast mitochondria"). We have also described a strategy aimed at circumventing this obstacle. Should our proposed strategy succeed, we will be able to use promoter strength and the affinity of the ribosome for the mRNA as tools for regulation of gene expression.

  Answers to these types of problems are currently being tested as part of the emerging field of Synthetic Biology within which, the Synberc initiative is becoming a main driving force. Since the project described here is part of this initiative we will look at the Synberc communitiy as a source of solutions for the problems we will encounter. The knowledge we will gain in the process of creating regulatory networks in the developing proto-cell will undoubtedly constitute the basis for the design of future synthetic organisms.

- Insufficient characterization of some mt-components or pathways. This is a long-term project and should take advantage of the increase in general knowledge pertaining mt-physiology occurring in parallel to its development. It is our intention to contribute to this knowledge whenever appropriate. We expect that the research conducted as part of this project will greatly increase our understanding of mt-physiology. To do this, we intend to form a group with a diverse disciplinary composition and to endow it with the means required for pursuing, when needed, both functional and structural characterization of mt-components. Moreover, we are planning on setting up collaborations with other research groups whenever necessary to solve individual aspects of the project.

CONCLUSION:

The studies proposed above are certainly both challenging and novel, but they fall, we believe, within the realm of what is now starting to be possible in biology. In the process of carrying out these studies, we will greatly increase our knowledge about mt-physiology, establish the foundations for the development of mt-gene therapy, and gain invaluable knowledge on how

to assemble and regulate large genomes.

We have made enormous progress during the last year towards achieving these goals. On the mammalian front we are much closer to achieving the delivery of DNA to the mitochondrial matrix. This accomplishment will not only pave the way for the realization of half of our proposed long term goals, i.e. reversing the endosymbiosis of mammalian mitochondria, but will also represent a biomedical milestone, as it will constitute the first step towards developing methods of mitochondrial gene therapy (Collombet, and Coutelle, 1998; D'Souza, *et al*, 2007). At the same time we are making a contribution to basic science, as we are pursuing the biochemical and structural characterization of the mt-components we are purifying in the lab.

On the yeast front, we have also made spectacular progress, as we are very close to having the first nuclear gene of our list integrated into the yeast mt-genome. Once this is confirmed, we will be able to study the consequences of endowing the mitochondria with increased autonomy from the nucleus. At the same time, we will enter a new dimension of the project aimed at developing tools to harness gene expression in the mt-matrix. Some of these tools are currently being tested.

Reference List

Alam, T.I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., Takio, K., Hamasaki, N., and Kang, D. (2003). Human mitochondrial DNA is packaged with TFAM. Nucleic Acids Res. *31,* 1640-1645.

Amiott, E.A., and Jaehning, J.A. (2006). Mitochondrial transcription is regulated via an ATP "sensing" mechanism that couples RNA abundance to respiration. Mol. Cell *22,* 329-338.

Andreoli, C., Prokisch, H., Hortnagel, K., Mueller, J.C., Munsterkotter, M., Scharfe, C., and Meitinger, T. (2004). MitoP2, an integrated database on mitochondrial proteins in yeast and man. Nucleic Acids Res. *32 Database issue,* D459-62.

Asin-Cayuela, J., Schwend, T., Farge, G., and Gustafsson, C.M. (2005). The human mitochondrial transcription termination factor (mTERF) is fully active in vitro in the non-phosphorylated form. J. Biol. Chem. *280,* 25499-25505.

Blanc, H., Adams, C.W., and Wallace, D.C. (1981). Different nucleotide changes in the large rRNA gene of the mitochondrial DNA confer chloramphenicol resistance on two human cell lines. Nucleic Acids Res. *9,* 5785-5795.

Bonawitz, N.D., Clayton, D.A., and Shadel, G.S. (2006). Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. Mol. Cell *24,* 813-825.

Bonnefoy, N., and Fox, T.D. (2002). Genetic transformation of Saccharomyces cerevisiae

mitochondria. Methods Enzymol. *350,* 97-111.

Brink, M.F., and de Boer, H.A. (1998). Analysis of rRNA function using specialized ribosomes. Methods Mol. Biol. *77,* 117-128.

Bunn, C.L., Wallace, D.C., and Eisenstadt, J.M. (1974). Cytoplasmic inheritance of chloramphenicol resistance in mouse tissue culture cells. Proc. Natl. Acad. Sci. U. S. A. *71,* 1681-1685.

Chang, D.D., and Clayton, D.A. (1986a). Identification of primary transcriptional start sites of mouse mitochondrial DNA: accurate in vitro initiation of both heavy- and light-strand transcripts. Mol. Cell. Biol. *6,* 1446-1453.

Chang, D.D., and Clayton, D.A. (1986b). Precise assignment of the heavy-strand promoter of mouse mitochondrial DNA: cognate start sites are not required for transcriptional initiation. Mol. Cell. Biol. *6,* 3262-3267.

Chang, D.D., and Clayton, D.A. (1986c). Precise assignment of the light-strand promoter of mouse mitochondrial DNA: a functional promoter consists of multiple upstream domains. Mol. Cell. Biol. *6,* 3253-3261.

Chang, D.D., and Clayton, D.A. (1984). Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA. Cell *36,* 635-643.

Chauhan, A., Tikoo, A., Kapur, A.K., and Singh, M. (2007). The taming of the cell penetrating domain of the HIV Tat: myths and realities. J. Control. Release *117,* 148-162.

Chuang, Y.Y., Chen, Q., Brown, J.P., Sedivy, J.M., and Liber, H.L. (1999). Radiation-induced mutations at the autosomal thymidine kinase locus are not elevated in p53-null cells. Cancer Res. *59,* 3073-3076.

Clark, M.A., and Shay, J.W. (1982). Mitochondrial transformation of mammalian cells. Nature *295,* 605-607.

Cliften, P.F., Jang, S.H., and Jaehning, J.A. (2000). Identifying a core RNA polymerase surface critical for interactions with a sigma-like specificity factor. Mol. Cell. Biol. *20,* 7013-7023.

Cohen, J.S., and Fox, T.D. (2001). Expression of green fluorescent protein from a recoded gene inserted into Saccharomyces cerevisiae mitochondrial DNA. Mitochondrion *1,* 181-189.

Collombet, J.M., and Coutelle, C. (1998). Towards gene therapy of mitochondrial disorders. Mol. Med. Today *4,* 31-38.

Costanzo, M.C., and Fox, T.D. (1988). Specific translational activation by nuclear gene products occurs in the 5' untranslated leader of a yeast mitochondrial mRNA. Proc. Natl. Acad. Sci. U. S. A. *85,* 2677-2681.

Del Gaizo, V., and Payne, R.M. (2003). A novel TAT-mitochondrial signal sequence fusion

protein is processed, stays in mitochondria, and crosses the placenta. Mol. Ther. *7,* 720-730.

Dietz, G.P., and Bahr, M. (2004). Delivery of bioactive molecules into the cell: the Trojan horse approach. Mol. Cell. Neurosci. *27,* 85-131.

D'Souza, G.G., Boddapati, S.V., and Weissig, V. (2007). Gene therapy of the other genome: the challenges of treating mitochondrial DNA defects. Pharm. Res. *24,* 228-238.

D'Souza, G.G., Boddapati, S.V., and Weissig, V. (2005). Mitochondrial leader sequence--plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria. Mitochondrion *5,* 352-358.

Dunstan, H.M., Green-Willms, N.S., and Fox, T.D. (1997). In vivo analysis of Saccharomyces cerevisiae COX2 mRNA 5'-untranslated leader functions in mitochondrial translation initiation and translational activation. Genetics *147,* 87-100.

Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G., and Gustafsson, C.M. (2002). Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nat. Genet. *31,* 289-294.

Fernandez-Silva, P., Enriquez, J.A., and Montoya, J. (2003). Replication and transcription of mammalian mitochondrial DNA. Exp. Physiol. *88,* 41-56.

Fernandez-Silva, P., Martinez-Azorin, F., Micol, V., and Attardi, G. (1997). The human mitochondrial transcription termination factor (mTERF) is a multizipper protein but binds to DNA as a monomer, with evidence pointing to intramolecular leucine zipper interactions. EMBO J. *16,* 1066-1079.

Fisher, R.P., Topper, J.N., and Clayton, D.A. (1987). Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements. Cell *50,* 247-258.

Gabaldon, T., and Huynen, M.A. (2004). Shaping the mitochondrial proteome. Biochim. Biophys. Acta *1659,* 212-220.

Gaspari, M., Falkenberg, M., Larsson, N.G., and Gustafsson, C.M. (2004). The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells. EMBO J. *23,* 4606-4614.

Gil, R., Silva, F.J., Pereto, J., and Moya, A. (2004). Determination of the core of a minimal bacterial gene set. Microbiol. Mol. Biol. Rev. *68,* 518-37, table of contents.

Glass, J.I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M.R., Maruf, M., Hutchison, C.A.,3rd, Smith, H.O., and Venter, J.C. (2006). Essential genes of a minimal bacterium. Proc. Natl. Acad. Sci. U. S. A. *103,* 425-430.

Golik, P., Bonnefoy, N., Szczepanek, T., Saint-Georges, Y., and Lazowska, J. (2003). The Rieske FeS protein encoded and synthesized within mitochondria complements a deficiency in

the nuclear gene. Proc. Natl. Acad. Sci. U. S. A. *100,* 8844-8849.

Heiser, W.C. (1994). Gene transfer into mammalian cells by particle bombardment. Anal. Biochem. *217,* 185-196.

Hutchison, C.A., Peterson, S.N., Gill, S.R., Cline, R.T., White, O., Fraser, C.M., Smith, H.O., and Venter, J.C. (1999). Global transposon mutagenesis and a minimal Mycoplasma genome. Science *286,* 2165-2169.

Jacob, W.F., Santer, M., and Dahlberg, A.E. (1987). A single base change in the Shine-Dalgarno region of 16S rRNA of Escherichia coli affects translation of many proteins. Proc. Natl. Acad. Sci. U. S. A. *84,* 4757-4761.

Jiao, S., Cheng, L., Wolff, J.A., and Yang, N.S. (1993). Particle bombardment-mediated gene transfer and expression in rat brain tissues. Biotechnology (N. Y) *11,* 497-502.

Kanki, T., Ohgaki, K., Gaspari, M., Gustafsson, C.M., Fukuoh, A., Sasaki, N., Hamasaki, N., and Kang, D. (2004). Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA. Mol. Cell. Biol. *24,* 9823-9834.

Karbowski, M., Kurono, C., Nishizawa, Y., Horie, Y., Soji, T., and Wakabayashi, T. (1997). Induction of megamitochondria by some chemicals inducing oxidative stress in primary cultured rat hepatocytes. Biochim. Biophys. Acta *1349,* 242-250.

Karbowski, M., Kurono, C., Wozniak, M., Ostrowski, M., Teranishi, M., Nishizawa, Y., Usukura, J., Soji, T., and Wakabayashi, T. (1999). Free radical-induced megamitochondria formation and apoptosis. Free Radic. Biol. Med. *26,* 396-409.

Kaufman, B.A., Durisic, N., Mativetsky, J.M., Costantino, S., Hancock, M.A., Grutter, P., and Shoubridge, E.A. (2007). The Mitochondrial Transcription Factor TFAM Coordinates the Assembly of Multiple DNA Molecules into Nucleoid-like Structures. Mol. Biol. Cell

Khan, S.M., and Bennett, J.P.,Jr. (2004). Development of mitochondrial gene replacement therapy. J. Bioenerg. Biomembr. *36,* 387-393.

Khan, S.M., Smigrodzki, R.M., and Swerdlow, R.H. (2007). Cell and animal models of mtDNA biology: progress and prospects. Am. J. Physiol. Cell. Physiol. *292,* C658-69.

King, M.P. (1996). Use of ethidium bromide to manipulate ratio of mutated and wild-type mitochondrial DNA in cultured cells. Methods Enzymol. *264,* 339-344.

King, M.P., and Attardi, G. (1989). Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science *246,* 500-503.

King, M.P., and Attardi, G. (1988). Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. Cell *52,* 811-819.

Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K.,

Ashikaga, S., Aymerich, S., Bessieres, P. *et al.* (2003). Essential Bacillus subtilis genes. Proc. Natl. Acad. Sci. U. S. A. *100,* 4678-4683.

Lecrenier, N., and Foury, F. (2000). New features of mitochondrial DNA replication system in yeast and man. Gene *246,* 37-48.

Lee, M., Choi, J.S., Choi, M.J., Pak, Y.K., Rhee, B.D., and Ko, K.S. (2007). DNA delivery to the mitochondria sites using mitochondrial leader peptide conjugated polyethylenimine. J. Drug Target. *15,* 115-122.

Luisi, P.L., Chiarabelli, C., and Stano, P. (2006). From never born proteins to minimal living cells: two projects in synthetic biology. Orig. Life Evol. Biosph. *36,* 605-616.

Martin, M., Cho, J., Cesare, A.J., Griffith, J.D., and Attardi, G. (2005). Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. Cell *123,* 1227-1240.

Matsuhashi, T., Liu, X., Nishizawa, Y., Usukura, J., Wozniak, M., and Wakabayashi, T. (1996). Mechanism of the formation of megamitochondria in the mouse liver induced by chloramphenicol. Toxicol. Lett. *86,* 47-54.

Mears, J.A., Cannone, J.J., Stagg, S.M., Gutell, R.R., Agrawal, R.K., and Harvey, S.C. (2002). Modeling a minimal ribosome based on comparative sequence analysis. J. Mol. Biol. *321,* 215-234.

Mireau, H., Arnal, N., and Fox, T.D. (2003). Expression of Barstar as a selectable marker in yeast mitochondria. Mol. Genet. Genomics *270,* 1-8.

Mitchell, D.J., Kim, D.T., Steinman, L., Fathman, C.G., and Rothbard, J.B. (2000). Polyarginine enters cells more efficiently than other polycationic homopolymers. J. Pept. Res. *56,* 318-325.

Mittelmeier, T.M., and Dieckmann, C.L. (1995). In vivo analysis of sequences required for translation of cytochrome b transcripts in yeast mitochondria. Mol. Cell. Biol. *15,* 780-789.

Mushegian, A.R., and Koonin, E.V. (1996). A minimal gene set for cellular life derived by comparison of complete bacterial genomes. Proc. Natl. Acad. Sci. U. S. A. *93,* 10268-10273.

Nakabachi, A., Yamashita, A., Toh, H., Ishikawa, H., Dunbar, H.E., Moran, N.A., and Hattori, M. (2006). The 160-kilobase genome of the bacterial endosymbiont Carsonella. Science *314,* 267.

Nass, M.M. (1969). Uptake of isolated chloroplasts by mammalian cells. Science *165,* 1128-1131.

Neupert, W., and Herrmann, J.M. (2007). Translocation of proteins into mitochondria. Annu. Rev. Biochem. *76,* 723-749.

Noireaux, V., Bar-Ziv, R., Godefroy, J., Salman, H., and Libchaber, A. (2005). Toward an

artificial cell based on gene expression in vesicles. Phys. Biol. *2,* P1-8.

Osteryoung, K.W., and Nunnari, J. (2003). The division of endosymbiotic organelles. Science *302,* 1698-1704.

Pizarro-Cerda, J., and Cossart, P. (2006). Subversion of cellular functions by Listeria monocytogenes. J. Pathol. *208,* 215-223.

Rackham, O., and Chin, J.W. (2005). A network of orthogonal ribosome x mRNA pairs. Nat. Chem. Biol. *1,* 159-166.

Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., and Pozzan, T. (1995). Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. Curr. Biol. *5,* 635-642.

Rizzuto, R., Nakase, H., Darras, B., Francke, U., Fabrizi, G.M., Mengel, T., Walsh, F., Kadenbach, B., DiMauro, S., and Schon, E.A. (1989). A gene specifying subunit VIII of human cytochrome c oxidase is localized to chromosome 11 and is expressed in both muscle and non-muscle tissues. J. Biol. Chem. *264,* 10595-10600.

Sato, A., Nakada, K., Akimoto, M., Ishikawa, K., Ono, T., Shitara, H., Yonekawa, H., and Hayashi, J. (2005). Rare creation of recombinant mtDNA haplotypes in mammalian tissues. Proc. Natl. Acad. Sci. U. S. A. *102,* 6057-6062.

Scheffler, I.E. (1999). Mitochondria (New York: A JOHN WILEY & SONS, INC., PUBLICATION).

Seo, B.B., Kitajima-Ihara, T., Chan, E.K., Scheffler, I.E., Matsuno-Yagi, A., and Yagi, T. (1998). Molecular remedy of complex I defects: rotenone-insensitive internal NADH-quinone oxidoreductase of Saccharomyces cerevisiae mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells. Proc. Natl. Acad. Sci. U. S. A. *95,* 9167-9171.

Seo, B.B., Wang, J., Flotte, T.R., Yagi, T., and Matsuno-Yagi, A. (2000). Use of the NADH-quinone oxidoreductase (NDI1) gene of Saccharomyces cerevisiae as a possible cure for complex I defects in human cells. J. Biol. Chem. *275,* 37774-37778.

Shine, J., and Dalgarno, L. (1974). The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. U. S. A. *71,* 1342-1346.

Steele, D.F., Butler, C.A., and Fox, T.D. (1996). Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc. Natl. Acad. Sci. U. S. A. *93,* 5253-5257.

Thyagarajan, B., Padua, R.A., and Campbell, C. (1996). Mammalian mitochondria possess homologous DNA recombination activity. J. Biol. Chem. *271,* 27536-27543.

Tiranti, V., Savoia, A., Forti, F., D'Apolito, M.F., Centra, M., Rocchi, M., and Zeviani, M. (1997). Identification of the gene encoding the human mitochondrial RNA polymerase (h-

mtRPOL) by cyberscreening of the Expressed Sequence Tags database. Hum. Mol. Genet. *6,* 615-625.

Tracy, R.L., and Stern, D.B. (1995). Mitochondrial transcription initiation: promoter structures and RNA polymerases. Curr. Genet. *28,* 205-216.

Weissig, V., Boddapati, S.V., Cheng, S.M., and D'Souza, G.G. (2006). Liposomes and liposome-like vesicles for drug and DNA delivery to mitochondria. J. Liposome Res. *16,* 249-264.

Weissig, V., Lasch, J., Erdos, G., Meyer, H.W., Rowe, T.C., and Hughes, J. (1998). DQAsomes: a novel potential drug and gene delivery system made from Dequalinium. Pharm. Res. *15,* 334-337.

Xu, B., and Clayton, D.A. (1995). A persistent RNA-DNA hybrid is formed during transcription at a phylogenetically conserved mitochondrial DNA sequence. Mol. Cell. Biol. *15,* 580-589.

Xu, R., Spencer, V.A., and Bissell, M.J. (2007). Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors. J. Biol. Chem. *282,* 14992-14999.

Yang, N.S., Burkholder, J., Roberts, B., Martinell, B., and McCabe, D. (1990). In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc. Natl. Acad. Sci. U. S. A. *87,* 9568-9572.

Yoon, Y.G., and Koob, M.D. (2005). Transformation of isolated mammalian mitochondria by bacterial conjugation. Nucleic Acids Res. *33,* e139.

Yoon, Y.G., and Koob, M.D. (2003). Efficient cloning and engineering of entire mitochondrial genomes in Escherichia coli and transfer into transcriptionally active mitochondria. Nucleic Acids Res. *31,* 1407-1415.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. (2000). An efficient recombination system for chromosome engineering in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. *97,* 5978-5983.

Yusupova, G., Jenner, L., Rees, B., Moras, D., and Yusupov, M. (2006). Structural basis for messenger RNA movement on the ribosome. Nature *444,* 391-394.